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PRINCIPAL INVESTIGATOR: Sarah Jenkins, Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham

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14. ABSTRACT

This grant investigated whether oral administration of the environmental contaminant Bisphenol A (BPA) could accelerate tumorigenesis in a transgenic mouse model that over-expresses the erbB2 proto-oncogene (erbB2-tg). BPA did not function in a traditional, linear dose responsive manner to induce tumorigenesis. Our data indicate that environmentally-relevant concentrations of BPA (BPA2.5 and BPA25) function in a distinctly different manner than regulatory-based concentrations of BPA (BPA250 and BPA2500). The environmentally-relevant BPA doses significantly accelerated mammary tumorigenesis, decreasing tumor latency and increasing tumor multiplicity, burden, and the rate of metastasis. These effects were absent in the regulatory-based BPA doses. Prior to tumor formation in the mammary gland, all doses of BPA significantly increased the cell proliferation index, but only the regulatory-based doses also increased the apoptosis index. When these two end points were used to estimate a ratio of cell-proliferation-to-apoptosis in the mammary gland, a non-monotonic dose response resulted. This end point provided the best prediction of effects induced by each concentration of BPA on mammary tumor development in erbB2-tg mice.

15. SUBJECT TERMS
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INTRODUCTION:

Bisphenol A (BPA) is a synthetically made compound used to produce a myriad of commonly used consumer goods. Studies have found that BPA is capable of leaching from these products in appreciable amounts, resulting in greater than 90% of study participants having detectable concentrations of urinary BPA. Due to BPA's endocrine disrupting effects and widespread exposure, it is suspected to play a role in breast cancer development and progression. We have previously shown that either prenatal (gestational days 10-21) only exposure to BPA or prepubertal (postpartum days 2-20) only exposure to BPA resulted in increased mammary cancer in a model of chemically-induced carcinogenesis. The purpose of this grant was to investigate the ability of BPA, via chronic oral administration, to accelerate tumorigenesis in a transgenic mouse model that over-expresses the erbB2 proto-oncogene (ErbB2-tg). Clinically, this mouse model represents an aggressive pathology of a large subset (15-30%) of human breast cancer cases.

BODY:

Aim 1a. To investigate the ontogeny of mammary pathology in erbB2-tg mice in order to identify the exact time to pathology under control conditions (Months 1-3)

The erbB2-tg mouse model was characterized under control conditions and set-up for use in this laboratory. Time points for subsequent aims were selected based on the ontogeny of mammary gland disease development and indices of cell proliferation and apoptosis prior to tumor development.

Sixteen weeks of age was selected as the end point for mechanistic studies. At this age, no preneoplastic/neoplastic lesions had been identified in the mammary glands of untreated mice. Also, untreated mice had not yet achieved peak or trough index values for either cell proliferation or apoptosis in the mammary gland. This window allowed us to assess either an increase or decrease in either index value as caused by BPA administration.

Thirty-six week of age was selected as the end point for the tumorigenesis study. At this age, there was an equal mix of well- and moderately-differentiated tumors identified in untreated mice. Further, at this age, there was no evidence of pulmonary metastasis in untreated mice. This allowed us to observe an unbiased change (either increase or decrease) in tumorigenesis in response to treatment with BPA.

Aim 1b. To identify the dose-dependent impact of BPA on mammary cancer progression in terms of palpable tumor latency, multiplicity, and pulmonary metastasis in a model of spontaneously developing mammary cancer (Months 1-8).

ErbB2-tg mice were given 0, 2.5 (BPA 2.5), 25 (BPA 25), 250 (BPA 250), or 2500 (BPA 2500) µg BPA/L drinking water or 54 ng estradiol/L (E2) drinking water. This was initiated at eight weeks of age and continued for the lifetime of the animal. It should be noted that the original proposal's lowest dose was set to BPA 25. However, the unusual circumstances of the first tumorigenesis study (non-monotonic dose response) urged us to perform a second tumorigenesis study which included an additional BPA dose that was a 10-fold reduction from the former lowest dose. This also provided another independent tumorigenesis study to validate and prove the robustness of the unusual response observed. Table 1 shows all of the BPA doses used, the resultant estimated intake in mice, and the significance of each dose. Broadly, these four doses can be divided into two categories, lower, environmentally relevant doses (BPA 2.5 and BPA 25) and higher, regulatory-based (BPA 250 and BPA 2500) doses of BPA.

Table 1. Estimated daily intake of BPA for erbB2-tg mice. Beginning at eight weeks of age, female erbB2-tg mice were provided 0, 2.5, 25, 250, or 2500 µg BPA/L drinking water. Estimated daily intakes of BPA were calculated on the basis of a 20 gram mouse drinking four milliliters of water daily.

Group	Treatment	Estimated Daily Intake	Significance
Control (n=94)	None	0 µg BPA/kg BW	Baseline
BPA 2.5 (n=38)	2.5 µg BPA/L	0.5 ug BPA/kg BW	Low Adult Exposure
BPA 25 (n=76)	25 µg BPA/L	5 µg BPA/kg BW	High Human Exposure
BPA 250 (n=37)	250 µg BPA/L	50 µg BPA/kg BW	EPA Reference Dose
BPA 2500 (n=36)	2500 µg BPA/L	500 µg BPA/kg BW	1% NTP's LOAEL

BPA did not elicit a traditional, linear dose-responsive effect on mammary carcinogenesis in erbB2-tg mice (Table 2). Only the lowest doses selected for use (BPA 2.5 and BPA 25) significantly accelerated tumorigenesis. The higher doses of BPA (BPA 250 and BPA 250) failed to differ significantly from control. The positive control, E2, also failed to significantly alter tumorigenesis.

The human relevant concentrations of BPA (BPA 2.5 and BPA 25) significantly increased tumor multiplicity, increasing the average number of tumors per mouse by 58% and 49%, respectively. Human relevant concentrations of BPA also significantly decreased the time-to-first-tumor latency, reducing the time of first tumor onset by over 29 days as compared to control. The time-to-second- and time-to-third-tumor latencies followed similar decreases (data not shown). Tumor volume was also significantly increased following chronic exposure to BPA 25 and trended towards an increase with BPA 2.5. Further, chronic exposure to human relevant concentrations of BPA significantly increased the percentage of mice that developed pulmonary metastasis. All of these effects were absent in the higher, regulatory-based doses of BPA.

Table 2. Tumor multiplicity, latency, burden, and the percent of animals with pulmonary metastasis following chronic, oral BPA exposure in erbB2-tg mice. Tumor multiplicity and burden are average values ± standard error of the mean.

Treatment	Tumor Multiplicity	Latency	Burden	% Metastasis
Control	1.03 ± 0.25	239 days	25.96 ± 3.12	7%
BPA 2.5	1.63 ± 0.25	210 days	36.00 ± 5.69	26%
BPA 25	1.52 ± 0.17	203 days	44.43 ± 5.92	18%
BPA 250	1.00 ± 0.16	240 days	25.08 ±6.47	11%
BPA 2500	0.97 ± 0.17	210 days	25.20 ± 6.33	5%
E2	1.06 ± 0.21	208 days	23.51 ± 5.50	3%

However, only the regulatory-based doses of BPA 250 and BPA 2500 altered body weight (Table 3). Both of these losses represented reductions in body weight less than 10%, which failed to reach toxicological significance. Chronic BPA administration also caused an increase in uterine wet weight and uterine to body weight ratio, with BPA 250 achieving statistical significance (Table 3). These data support existing evidence in the literature that BPA functions as a weak estrogen only at high concentrations not likely to be achieved in “normal” human situations. While the E2 positive control significantly decreased body weight, it failed to significantly increase uterine wet weight or the uterine-to-body weight ratio (Table 3).

Table 3. Body weight, uterine wet weight, and uterine-to-body-weight ratio in erbB-tg mice following chronic oral exposure to BPA. Values represent averages ± standard error. As compared to control, * indicates a p-value <0.05, and *** indicates a p-value <0.001.

Treatment	Body Weight (g)	Uterine Weight (mg)	Uterine:BW Ratio
Control	26.0 ± 0.3	90.2 ± 4.3	3.5 ± 0.2
BPA 2.5	25.3 ± 0.6	100.7 ± 7.2	4.0 ± 0.3
BPA 25	25.9 ± 0.4	107.3 ± 6.0	4.2 ± 0.2
BPA 250	24.1 ± 0.2*	123.3 ± 13.6*	5.1 ± 0.6***
BPA 2500	24.4 ± 0.3	113.0 ± 6.5	4.6 ± 0.3
E2	24.00 ±0.35*	104.34 ± 5.75	4.37 ± 0.24

However, not all end points in this aim were significantly altered. There were no statistically significant changes between any of the treatment groups for tumor incidence (data not shown). Similarly, there were no statistically significant differences in tumor grade, the presence of necrosis, or in the tissue of origin (data not shown). No significant differences were found in either the percentage

of data censored for each group or in the number of animals sacrificed prior to the end of the tumorigenesis study (252 days of age). All resultant tumors included in the tumorigenesis study were pathologically confirmed to be invasive carcinoma of mammary origin by a board-certified pathologist, Dr. Isam Eltoum.

Aim 2: To determine the mechanism of action behind BPA's effects on promoting mammary carcinogenesis in erbB2 over-expressing mice.

Task 2.4: Apoptosis & Proliferation

All doses of BPA investigated increased the index of cell proliferation in the mammary gland of erbB2-tg mice (Table 4). However, only the higher, regulatory-based doses of BPA increased the index of apoptosis in the mammary gland, with BPA 2500 achieving statistical significance. It is interesting to note that when the ratio of cell proliferation index to apoptosis index was calculated, a non-monotonic dose response resulted. It was this curve that best predicted the response of the erbB2-tg mouse model to each dose of BPA. BPA 2.5 and BPA 25 had an increased proliferation-to-apoptosis ratio, with BPA 25 being statistically significant as compared to control. While the E2 positive control significantly increased the cell proliferation, it failed to significantly alter the apoptotic index. The details of these data can be found in Table 4.

Table 4. Proliferation, apoptosis, and the ratio of cell proliferation to apoptosis ratio in the mammary glands of erbB2-tg mice treated with BPA. Female erbB2-tg mice were exposed to 0 (n=15), 2.5 (n=8), 25 (n=8), 250 (n=8), and 2500 (n=8) µg BPA/L drinking water or 54 ng E2/L (n=8) drinking water from eight until 16 weeks of age. * Represents a statistically significant p-value ($p<0.05$) as compared to control.

Treatment	Proliferation Index	Apoptosis Index	Prolif:Apoptosis Ratio
Control	0.08 ± 0.01	0.007 ± 0.001	20.29 ± 4.84
BPA 2.5	0.18 ± 0.03	0.004 ± 0.001	48.33 ± 11.07
BPA 25	0.33 ± 0.03*	0.005 ± 0.002	153.06 ± 54.79*
BPA 250	0.30 ± 0.03*	0.015 ± 0.004	20.22 ± 6.41
BPA 2500	0.30 ± 0.06*	0.031 ± 0.008*	21.42 ± 10.59
E2	0.21 ± 0.04*	0.010 ± 0.004	9.40 ± 2.11

Task 2.5: IHC Staining

For the remainder of this aim, selective doses from each category of exposure (environmentally-relevant (BPA 25) and regulatory-related (BPA 2500) were selected. Immunohistochemical staining was carried out for the major female sex steroid receptors, estrogen receptor alpha (ER alpha), estrogen receptor beta (ER beta), progesterone receptor a (PR-A), and progesterone receptor b (PR-B) (Table 5). At 16 weeks of age, ER alpha exhibited significantly decreased expression in the mammary glands of BPA 25 treated mice as compared to

control. Interestingly, while the expression of ER alpha in BPA 2500 treated mice failed to differ significantly from control, it was significantly increased as compared to BPA 25 ($p<0.05$). A similar pattern of expression was observed for ER beta.

To ensure that this down-regulation of the ERs was not caused by excessive ER activation and subsequent degradation, we assessed the expression of PR-A, a known downstream target of classical ER signaling. Chronic exposure to BPA 25 resulted in similarly decreased expression of PR-A in the mammary gland as compared to control treated mice ($p<0.01$). This effect was not observed for the BPA 2500 treatment group, which failed to differ significantly from control. However, BPA 2500 exhibited significantly increased PR-A expression as compared to BPA 25 ($p<0.01$). Sustained exposure to BPA failed to alter the expression of PR-B.

Table 5. Immunohistochemical staining of ER alpha, ER beta, PR-A, and PR-B at 16 weeks of age in erbB2-tg mice treated with BPA. * Represents a statistically significant p-value ($p<0.05$) as compared to control. ^ Represents a statistically significant p-value ($p<0.05$) as compared to BPA 25.

Treatment	ER Alpha	ER Beta	PR-A	PR-B
Control	0.28 ± 0.002	0.53 ± 0.06	0.40 ± 0.03	0.04 ± 0.02
BPA 25	0.19 ± 0.02*	0.29 ± 0.07*	0.25 ± 0.02*	0.01 ± 0.01
BPA 2500	0.31 ± 0.04^	0.51 ± 0.07	0.37 ± 0.05^	0.01 ± 0.01

Task 2.6: Western Blotting

Given our findings concerning BPA-induced changes of the expression of ER alpha, ER beta, and PR-A, the first major signaling pathway selected for investigation via western blotting was that of the p160 family of steroid receptor coactivators (SRC) (Table 6). SRC-3 (also known as amplified in breast cancer 1 or AIB1) was significantly up-regulated in both BPA 25 ($p<0.01$) and BPA 2500 ($p<0.01$) treatment groups as compared to control (Figure 17B). Expression of the two remaining members, SRC-1 and SRC-2, both failed to differ between any of the treatment groups.

Table 6. Immunoblot analysis of SRC-1, SRC-2, and SRC-3/AIB1 at 16 weeks of age in erbB2-tg mice treated with BPA. The table illustrates mean density ± standard error of the mean as a percent of the control group. * Represents a statistically significant p-value ($p<0.05$) as compared to control.

Treatment	SRC-1	SRC-2	SRC-3/AIB1
Control	100 ± 3.5	100 ± 8.8	100 ± 10.4
BPA 25	106.8 ± 3.5	91.1 ± 11.1	176.9 ± 11.1*

BPA 2500	106.8 ± 3.9	105.8 ± 14.0	169.5 ± 9.2*

Next, we examined the erbB and insulin-like growth factor (IGF) tyrosine kinase growth factor signaling pathways as both have been documented to play major roles in the development of pathology in the erbB2-tg mouse model (Figure 1; Figure 2). No differences were observed in the protein expression of erbB2 between any of the treatment groups (Figure 1). This indicates that the accelerated carcinogenesis observed was not due to BPA simply increasing expression of the transgene. Despite this, BPA 25, but not BPA 2500, resulted in significantly increasing the activation of erbB2 ($p<0.05$) as compared to control. While BPA 25 significantly decreased the expression of erbB3 as compared to BPA 2500 treated mice, it was still capable of significantly increasing erbB3 activation as compared to control ($p<0.05$). The protein expression of erbB1/EGFR was unaltered by any of the treatment groups. Measuring the expression of the erbB ligand reported to be under the control of ER action, amphiregulin, we found a dose-dependent increase in protein expression. Accordingly, BPA 2500 exhibited a significant increase in the expression of amphiregulin as compared to control ($p<0.05$) and BPA 25 ($p<0.05$).

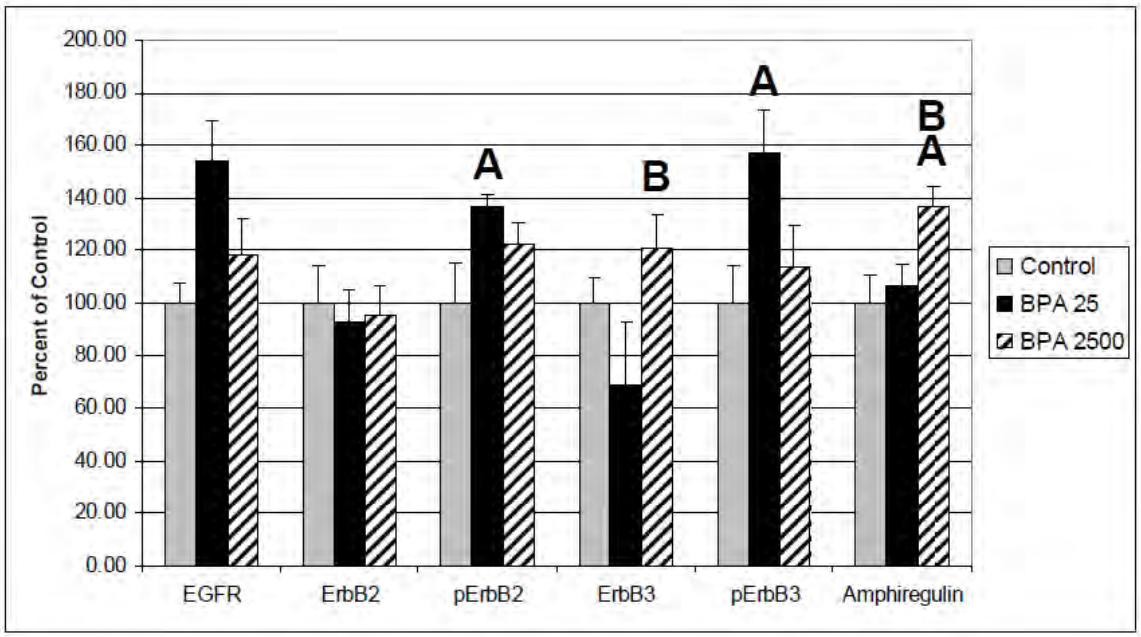

Figure 1. Protein expression of EGFR, erbB2, phospho-erbB2, erbB3, phospho-erbB3, and amphiregulin in the mammary glands of erbB2-tg mice exposed to 0, 25, or 2500 µg BPA/L drinking water. Each treatment contained 6-8 samples. The graphs illustrate mean density ± standard error of the mean as a percent of the control group. A p-value ≤ 0.05 was considered statistically significant. [A] represents a statistically significant p-value as compared to control. [B] represents a statistically significant p-value as compared to BPA 25.

The BPA 25 treatment group significantly up-regulated the expression and activation of IGF-1 receptor as compared to both control and BPA 2500 (Figure

2). We investigated the local expression of the IGF-1 receptor ligand, IGF-1, in the mammary gland as opposed to circulating values found in the serum. BPA 25 treatment was capable of significantly increasing the local mammary gland expression of IGF-1 ligand as compared to control ($p<0.05$) but not BPA 2500 treated mice.

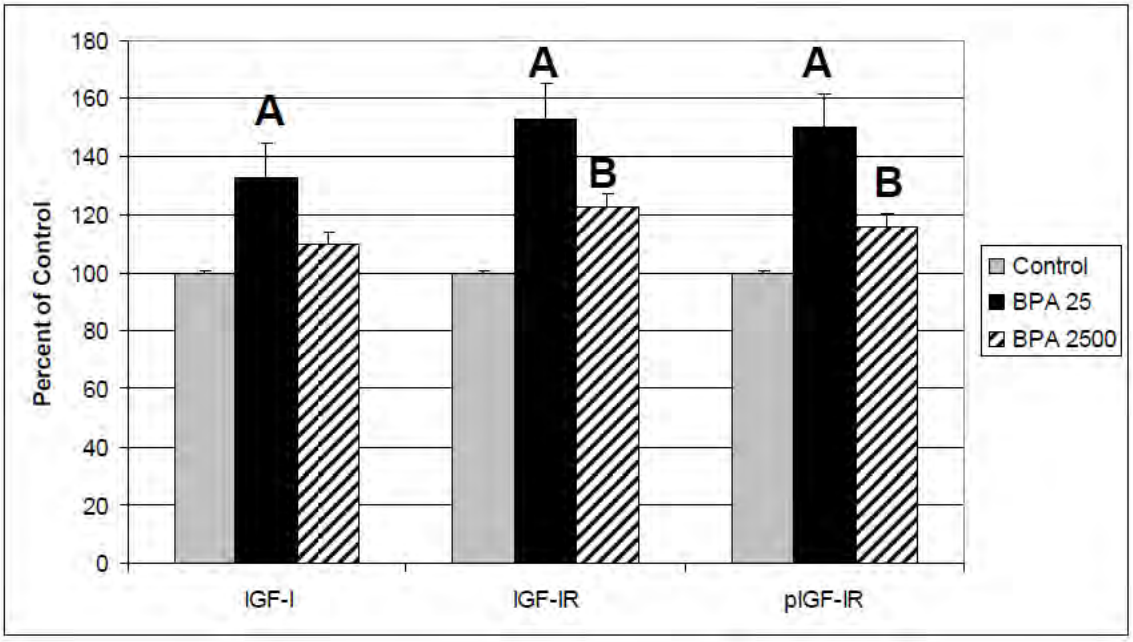

Figure 2. Protein expression of IGF-1, IGF-IR, and phospho-IGF-IR in the mammary glands of erbB2-tg mice exposed to 0, 25, or 2500 µg BPA/L drinking water. Each treatment contained 6-8 samples. The graphs illustrate mean density ± standard error of the mean as a percent of the control group. A p-value ≤ 0.05 was considered statistically significant. [A] represents a statistically significant p-value as compared to control. [B] represents a statistically significant p-value as compared to BPA 25.

ErbB3 contains six docking sites for phosphoinositol (3,4,5)-triphosphate (PIP3), which serves as a docking site for the serine kinase, Akt (protein kinase B). Heterodimerization between erbB2 and erbB3 has been reported to induce preferential and potent activation of the Akt pathway.

The expression of PI3K and PTEN, which function as opposing gatekeepers for the activation of Akt, in the mammary gland did not differ significantly between any of the treatment groups at 16 weeks of age (Figure 3). BPA 25 treatment significantly increased the expression of the Akt 1 isoform as compared to control ($p<0.01$) and the Akt 3 isoform as compared to BPA 2500 ($p<0.05$). Activation of Akt (pan-activation), as measured by the protein concentration of phosphorylated Akt, was significantly up-regulated by BPA 25 as compared to control ($p<0.01$) and BPA 2500 ($p<0.05$).

In addition, several downstream proteins involved in this pathway were regulated, including glycogen synthase kinase 3 beta (GSK-3ß), Bad, and the extracellular signal-regulated kinases (ERKs 1 and 2) (Figure 3). Normally involved in the inhibition of glycogen synthesis and tagging the oncogenic beta catenin for degradation, GSK-3ß is phosphorylated and consequently inactivated by Akt. BPA 25 resulted in significantly increasing the phosphorylation of GSK-3ß (inactive) as compared to control ($p<0.0001$) and BPA 2500 ($p<0.001$). This increased inactivation was not due to increased expression of GSK-3ß (active), which was unaltered by any of the treatment groups (data not shown).

Bad, which normally functions as a pro-apoptotic protein, also becomes inactivated when phosphorylated by Akt. BPA 25 ($p<0.0001$) and BPA 2500 ($p<0.01$) significantly up-regulated the phosphorylation (inactivation) of Bad as compared to control. The ERKs are involved in downstream signaling initiated by both the erbB and IGF family of receptor tyrosine kinases and have been shown to play integral roles in cellular proliferation. The activation of ERK 1 and ERK 2 was up-regulated in response to BPA 25, but not BPA 2500, treatment.

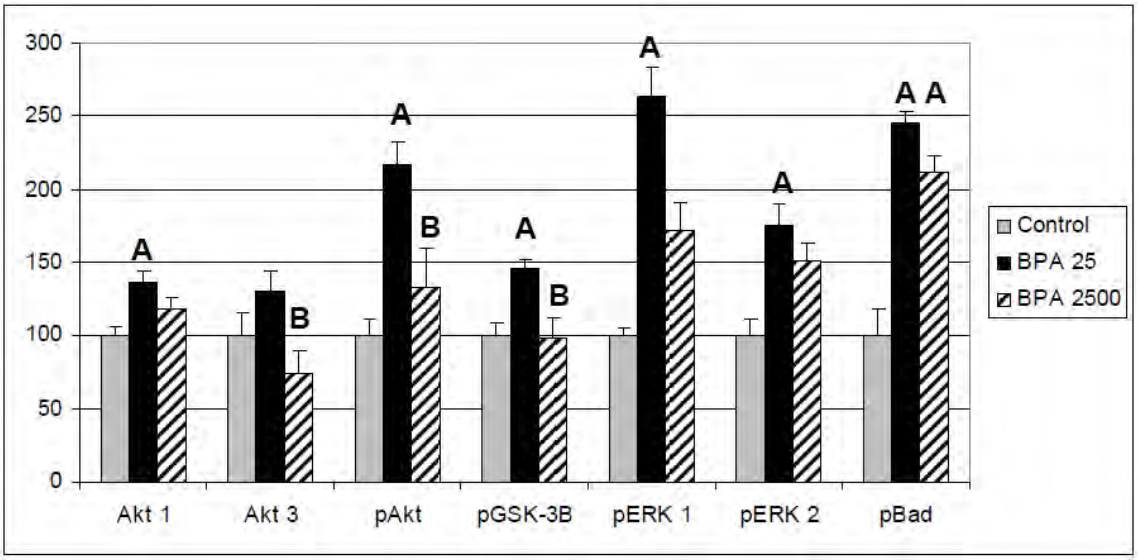

Figure 3. Protein expression of Akt 1, Akt 3, phospho-Akt, phospho-GSK-3 beta, phospho-ERK 1, phospho-ERK 2, and phospho-Bad in the mammary glands of erbB2-tg mice exposed to 0, 25, or 2500 µg BPA/L drinking water. Each treatment contained 6-8 samples. The graphs illustrate mean density ± standard error of the mean as a percent of the control group. A p-value ≤ 0.05 was considered statistically significant. ^{A} represents a statistically significant p-value as compared to control. ^{B} represents a statistically significant p-value as compared to BPA 25.

Chronic exposure to BPA resulted in a dose-dependent increase in cellular proliferation in the mammary gland. This increased rate of proliferation was countered by an increased rate of apoptosis by some, but not all, of the BPA treatment groups. Specifically, while the higher, regulatory-based doses of BPA

(BPA 250 and BPA 2500) were capable of countering an increased index of cellular proliferation with an increased index of apoptosis in the mammary gland, the lower, environmentally-relevant doses of BPA (BPA 2.5 and BPA 25) lacked this response. This resulted in an altered rate of cell turnover in BPA 2.5 and BPA 25 that did not exist in BPA 250 and BPA 2500. Accordingly, we investigated several key proteins found to play important roles in the regulation of apoptosis.

Of particular interest were the dose-specific alterations in caspase activation. Increased activation of caspase 2 and caspase 9 was observed in response to BPA 2500 only (Figure 4). While the activation of caspase 8 was significantly increased in BPA 2500 as compared to BPA 25 treated mice, it failed to differ significantly from control. BPA 25 significantly down-regulated the activation of caspase 8 as compared to control. Interestingly, caspase 3 activation was significantly decreased by BPA 2500 treatment when compared to both control and BPA 25.

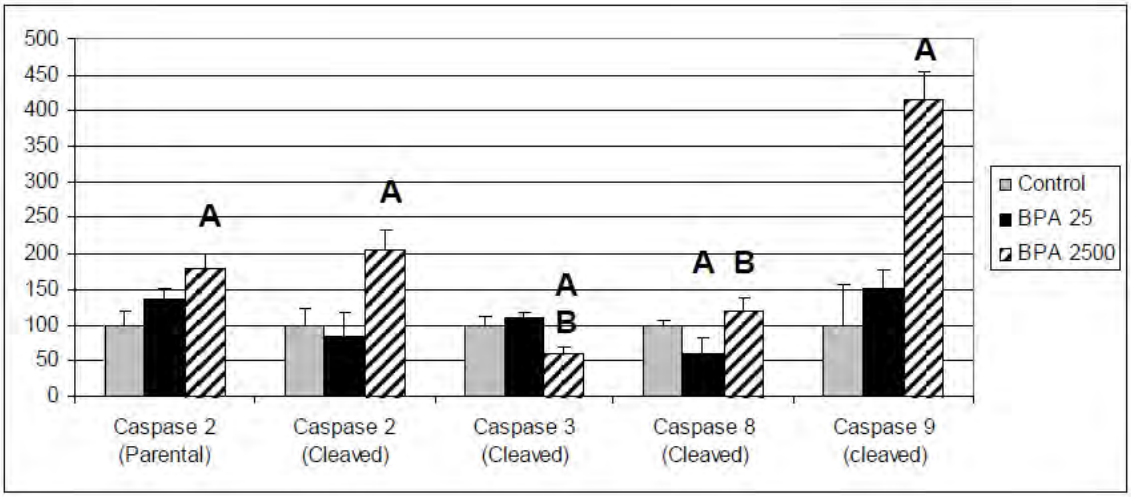

Figure 4. Protein expression of caspase 2, cleaved caspase 2, cleaved caspase 3, cleaved caspase 8, and cleaved caspase 9 in the mammary glands of erbB2-tg mice exposed to 0, 25, or 2500 µg BPA/L drinking water. Each treatment contained 6-8 samples. The graphs illustrate mean density ± standard error of the mean as a percent of the control group. A p-value ≤ 0.05 was considered statistically significant. ^{A} represents a statistically significant p-value as compared to control. ^{B} represents a statistically significant p-value as compared to BPA 25.

Task 2.7: 2D Gels

Female erbB2-tg mice were exposed to the most carcinogenic concentration of BPA, BPA 25, or vehicle from eight until 16 weeks of age. During the estrous phase, the fourth abdominal mammary glands were dissected out and immediately snap frozen in liquid nitrogen. Whole mammary glands were homogenized in liquid nitrogen, subjected to albumin and IgG removal and acetone precipitated, before being loaded onto immobilized pH gradient strips

(pH 4-7). Proteins were separated according to isoelectric point in the first dimension and by molecular weight in the second dimension. Gels were fixed, stained with SyproRuby overnight, destained, and scanned to produce high quality images for use with the 2D gel analysis software, SameSpot.

While over 3,000 unique spots were identified, statistical analysis identified only five of these spots as being differentially regulated in the mammary glands of BPA 25 treated mice as compared to control. These gel plugs were manually excised, subjected to trypsin digestion, and identified through a combination of MALDI-TOF/TOF and LC-MS. The four proteins that were identified from these gel plugs are summarized in Table 7. Unfortunately, these proteins solely represent high abundance proteins with little implication to the development of pathology.

Table 7: Putative protein identifications by mass spectrometry, resultant p-values, and fold change from 2D gel spots determined to be significantly regulated by BPA 25 in erbB2-tg mice as compared to control.

Spot ID	Protein ID	p-value	Fold Change
74	Protein Disulfide Isomerase A6	0.017	-1.3
52	Clp Protease Proteolytic Subunit	0.014	-1.7
73	Chloride Intracellular Channel Protein 1	0.02	-1.3
67	Proteasome Subunit Alpha	0.04	-1.4

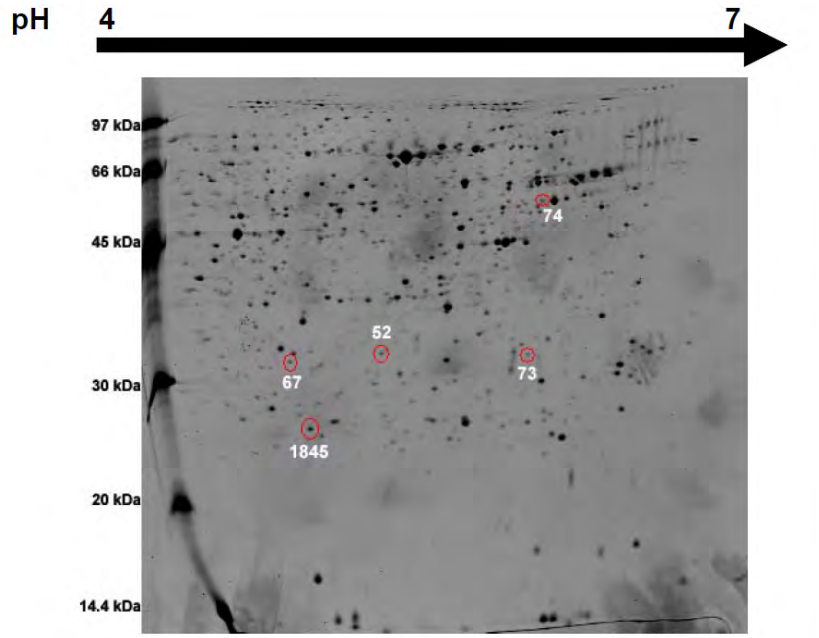

Figure 5. A representative 2D gel. The red circles indicate protein spots that were found to be differentially regulated and identified by mass spectrometry as identified in Table 7.

Since their advent in the 1970s, 2D gels have been one of the most used methods for protein fractionation. 2D gels function by harnessing the chemical and structural differences inherent to proteins, namely the isoelectric point and molecular weight. Being paired with mass spectrometry for protein identification, they represent a robust and well developed method for global analysis of a large subsection of the proteome. Unlike the limited resolving power of simply separating protein lysate according to molecular weight as in traditional SDS-PAGE, 2D gels allow for the ability to resolve thousands of proteins in a single gel. It provides a technology relatively easy to implement in most laboratories and can be used in the qualitative and quantitative analysis of a large subset of the proteome. We have previously used this method to successfully assess changes in protein expression patterns that can serve as biomarkers of exposure and that have provided insight into novel pathways affects by xenoestrogen exposures.

However, in the case of the present research, we were unable to link any plausible mechanism of action between BPA and carcinogenesis from the identity of these proteins. There are several potential reasons for this. There is a limited sample capacity for 2D gels. Low molecular weight and low abundance proteins are rarely visualized due to lack of sensitivity and masking by high abundance proteins. Small changes in protein expression are not easily recognized. The mammary gland is a complex organ composed of several tissue types aside from our key target, epithelial cells. Methods to break the tissue down according to tissue type, such as laser capture microdissection or enzymatic digestions with collagenase, do not provide realistic alternatives. Laser capture microdissection is ill suited for protein work, requiring an inordinate number of cells being used in order to obtain the required protein concentrations. Attempts to separate the mammary gland into epithelial and stromal cell compartments based on collagenase digestions proves to be time consuming, requires several mice be pooled into a singular sample, and can potentially add greater sample-to-sample variability depending on what epithelial structures (TEBs, TDs, lobuloalveolar buds, etc.) are selected.

One alternative would be to apply 2D gels to a simpler, *in vitro* model of BPA exposure. Other alternatives involve utilizing newer, non-gel based proteomic technologies. Ex-*vivo* peptide tagging methods, such as isotope coded affinity tags (ICAT) or isobaric tag for relative and absolute quantitation (ITRAQ) both represent attractive possibilities for future studies.

Aim 3: To measure circulating concentrations of BPA in erbB2-tg mice after sub-chronic (16 weeks of age) and chronic (36 weeks of age, 28 weeks of treatment) administration of BPA.

ErbB2-tg mice were given 0, 25, 250, or 2500 µg BPA/L drinking water beginning at eight weeks of age. At 16 and 36 weeks, mice were sacrificed and blood was collected via cardiac puncture. The blood samples were allowed to clot at room

temperature, and serum was collected after slow speed centrifugation. In all instances, a sufficient volume for analysis required the pooling of serum samples from several mice. However, at each time point, there were a minimum of eight pooled samples per treatment group. Table 8 describes the data obtained after sub-chronic treatment (16 weeks of age, 8 weeks on treatment) with BPA. Table 9 describes the data obtained from chronic treatment (36 weeks of age, 28 weeks on treatment) with BPA.

Table 8. Free and total concentrations of BPA in the serum of erbB2-tg mice at 16 weeks of age (8 weeks on treatment).

Treatment	Avg. Free BPA (ng/ml)	Avg. Total BPA (ng/ml)
Control	0.9 ± 0.2	0.9 ± 0.3
BPA 25	9.0 ± 5.5	9.0 ± 5.4
BPA 250	1.0 ± 0.2	1.7 ± 0.3
BPA 2500	0.7 ± 0.1	12.2 ± 1.5

Table 9. Free and total concentrations of BPA in the serum of erbB2-tg mice at 36 weeks of age (28 weeks on treatment).

Treatment	Avg. Free BPA (ng/ml)	Avg. Total BPA (ng/ml)
Control	0.9 ± 0.2	0.9 ± 0.3
BPA 25	9.0 ± 5.5	9.0 ± 5.4
BPA 250	1.0 ± 0.2	1.7 ± 0.3
BPA 2500	0.7 ± 0.1	12.2 ± 1.5

KEY RESEARCH ACCOMPLISHMENTS

Aim 1a: Ontogeny Study of the ErbB2-tg Mouse Model

- Sixteen and 36 weeks of age were selected for all downstream mechanistic and tumorigenesis studies, respectively.

Aim 1b: BPA Tumorigenesis in ErbB2-tg Mice

- BPA 2.5 and BPA 25 significantly accelerated tumorigenesis by decreasing tumor latency and increasing tumor multiplicity, tumor burden, and the incidence of pulmonary metastasis.
- There was a dose-dependent increase in uterine wet weight both alone and when corrected for body weight. However, these effects were only significant at the highest doses administered, supporting the prevalent thinking that BPA is a weak estrogen and behaves as a classical estrogen only at high concentrations.
- BPA caused a dose-dependent decrease in body weight, with BPA 2500 achieving statistical, but not toxicological, significance.

Aim 2: BPA's Mechanism of Action

- All doses of BPA increased the index of cell proliferation in the mammary gland. However, only the regulatory-based doses of BPA increased the apoptosis index in the mammary gland, with BPA 2500 achieving statistical significance. This resulted in an altered ratio of cell proliferation in the low, environmentally relevant concentrations of BPA but not in the regulatory-based concentrations of BPA.
- Using selective doses from each category of exposure (BPA 25 for environmentally relevant doses and BPA 2500 for regulatory-based doses), we found significant differences at the protein level to suggest that BPA's mechanism of action is a function of the administered dose. BPA 25 significantly reduced the expression of ER alpha, ER beta, PR-A and increased expression of phospho-erbB2, phospho-erbB3, IGF-1, IGF-1R, phospho-IGF-1R, Akt. These effects were absent from BPA 2500.
- The primary pathway we identified as being altered by BPA 25, but not BPA 2500, was the PI3k/Akt signaling pathway. BPA 25 significantly regulated the expression and/or activation of Akt, GSK, and the ERKs.
- However, BPA 2500, but not BPA 25, regulated a select number of proteins involved in apoptosis, including caspase 2, cleaved caspase 2, caspase 3, and cleaved caspase 9. These findings add merit to the idea that BPA 2500 is able to avoid accelerating mammary carcinogenesis by also increasing the incidence of apoptosis.

Aim 3: Circulating Concentrations of BPA

- Both subchronic and chronic administration of BPA largely resulted in low, circulating concentrations of BPA which were similar to reported human values.

REPORTABLE OUTCOMES

Degrees: Ph.D. in Pharmacology and Toxicology

Awards: (1) Cancer Prevention and Control Fellowship, (2) Society of Toxicology Student Travel Award, (3) Susan G. Komen Travel Scholarship, (4) 3rd Place in UAB's Graduate Student Research Days Presentation, and (5) William C. Bailey Award for Excellence in Cancer Prevention and Control.

Abstracts:

Jenkins S, Kennerly R, and Lamartiniere CA. Bisphenol A & the Non-Monotonic Dose Response: Why Environmentally Relevant Doses Pose More Danger than Pharmacological Doses. IUTOX (Barcelona, Spain) in July 2010

Jenkins S, Kennerly R, and Lamartiniere CA. Bisphenol A & the Non-Monotonic Dose Response: Why Environmentally Relevant Doses Pose

More Danger than Pharmacological Doses. PPTOXII (Miami Beach, FL). December 2009.

Lamartiniere CA, **Jenkins S**, Bentancourt AB. Early BPA Exposure Alters the Proteome and Susceptibility for Mammary Cancer in Rodents. The Role of Environmental Stressors in the Developmental Origins of Disease pp. V.12. Miami, FL (2009)

Jenkins S, Kennerly R, and Lamartiniere CA. Bisphenol A & the Non-Monotonic Dose Response: Why Environmentally Relevant Doses Pose More Danger than Pharmacological Doses. UAB Comprehensive Cancer Center Research Retreat (Birmingham, AL). October 2009.

Jenkins S and Lamartiniere CA. Sustained Exposure to Low Doses of BPA Results in a Non-Monotonic Dose Response Curve of Tumorigenesis in the MMTV-erbB2 Transgenic Mouse Model. Gordon Conference: Hormone Action in Development & Cancer (Holderness, NH) July 2009.

Jenkins S, Betancourt A, Mobley J, Lamartiniere CA. Chronic Bisphenol A Exposure Accelerates Mammary Tumorigenesis and Alters the Mammary Proteome. 3rd European Proteomics Association Congress (Stockholm, Sweden) June 2009.

Jenkins S, Lamartiniere CA. Chronic Bisphenol A Exposure during Adulthood Accelerates Mammary Carcinogenesis. Breast Cancer and the Environment Research Centers' Fifth Annual Early Environmental Exposure Meeting (Birmingham, AL), November 2008.

Jenkins S, Raghuraman N, Lamartiniere CA. Early Exposure to Bisphenol A Imparts Increased Tumorigenesis and Long-Lasting Alterations to the Protein Expression in the Mammary Glands of Adult Rats. Breast Cancer and the Environment Research Centers' Fifth Annual Early Environmental Exposure Meeting (Birmingham, AL), November 2008.

Jenkins S, Lamartiniere CA. Evaluating the Impact of Adult Exposure to Bisphenol A on Women with Breast Cancer. Breast Cancer and the Environment Research Centers' Fifth Annual Early Environmental Exposure Meeting (Birmingham, AL), November 2008.

Jenkins S, Lamartiniere CA. Adult Exposure to the Plastic Component, Bisphenol A, Accelerates ErbB2-Positive Mammary Cancer in Mice. UAB Comprehensive Cancer Center Research Retreat (Birmingham, AL). November 2008.

Oral Presentations:

Jenkins S and Lamartiniere CA (2009) *Chronic Exposure to the Plastic Component, Bisphenol A, Accelerates Mammary Carcinogenesis.* February 2009. Department of Pharmacology & Toxicology seminar series, UAB.

Jenkins S and Lamartiniere CA (2009) *Chronic Exposure to the Plastic Component, Bisphenol A, Accelerates ErbB2-Positive Mammary Cancer*. February 2009. Graduate Student Research Days, UAB.

Manuscripts: See appendices for full text.

Lamartiniere CA, **Jenkins S**, Betancourt AM, and Wang J (2010) Exposure to the Endocrine Disruptor Bisphenol A Alters Susceptibility for Mammary Cancer (2010). (**Accepted for Publication**)

Jenkins S, , Betancourt AM, Wang J, Mobley JA, and Lamartiniere CA. Proteomic Basis for the Increased Susceptibility of the Mammary Gland to Carcinogenesis after Perinatal Exposure to Bisphenol A. Edited by Jose Russo. (**In Submission**)

Jenkins S, Eltoum I, Desmond R, and Lamartiniere CA. Chronic Oral Exposure to Environmentally Relevant Concentrations of Bisphenol A Increases Mammary Carcinogenesis and Metastasis. **(In Submission)**

CONCLUSIONS

When BPA is administered orally to erbB2-tg mice, it does not function in a traditional, linear dose-responsive manner to induce mammary tumorigenesis. Instead, it was only with the lower doses of BPA studied (BPA 2.5 and BPA 25), those doses which are achievable through normal dietary intake, that were capable of significantly accelerating mammary tumorigenesis in this model. The higher, regulatory based doses of BPA (BPA 250 and BPA 2500) and the E2 positive control failed to significantly alter tumorigenesis. However, these regulatory-based treatment groups and E2 did significantly alter overt markers of toxicity, which could have contributed to a lack of tumorigenic response.

Out data indicate that the environmentally relevant doses of BPA function in a distinctly different manner than the regulatory-based doses at the cellular level. While all doses of BPA increased the index of cell proliferation, it was only the regulatory-based doses of BPA that increased the rate of apoptosis. This was further supported by the regulatory-based dose, but not the environmentally-relevant dose, altering proteins involved with apoptosis. Because the environmentally relevant doses of BPA failed to increase the index of apoptosis in the mammary gland to counter the high index of cell proliferation, a

significantly altered ratio of “cell turnover” (cell proliferation:apoptosis ratio) resulted. It was this ratio which best predicted the tumorigenic response of the erbB2-tg mouse model to each dose of BPA studied.

Further, we found that representative doses from each category of exposure (environmental vs. regulatory) regulated different proteins in the mammary gland. BPA 25, which was the most tumorigenic dose of BPA administered in this study, significantly down-regulated the expression of ER alpha, ER beta, and PR-A while significantly up-regulating the expression of phospho-erbB2, phospho-erbB3, phospho-IGF-1R, Akt1, phospho-Akt, phospho-GSK-3B, and phospho-ERK ½. This pattern suggests that environmentally relevant concentrations of BPA may function to increase mammary carcinogenesis through the aberrant activation of erbB and IGF growth factor receptors and the downstream Akt pathway. On the other hand, BPA 2500 significantly up-regulated several members of the caspase family. This suggests that BPA 2500 may avoid increased carcinogensis by inducing a countering increase in apoptosis in the mammary gland.

To address the training aspects of this grant, I have: presented 10 posters at international and national meetings, given two oral presentations, published two manuscripts and have two additional manuscripts submitted for publication, won several travel awards and poster competitions, and have successfully matriculated with my doctoral degree in Pharmacology and Toxicology.

REFERENCES: none

APPENDIX A:

Lamartiniere CA, **Jenkins S**, Betancourt AM, and Wang J (2010) Exposure to the Endocrine Disruptor Bisphenol A Alters Susceptibility for Mammary Cancer (2010). (**Accepted for Publication**

Exposure to the Endocrine Disruptor Bisphenol A Alters Susceptibility for Mammary Cancer

Coral A. Lamartiniere[1,2], Sarah Jenkins[1], Angela M. Betancourt[1], Jun Wang[1] and Jose Russo[3]

[1]Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, Alabama

[2]UAB Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama

[3]Breast Cancer Research Laboratory, Fox Chase Cancer Center, Philadelphia, PA

Corresponding Author: Coral A. Lamartiniere, Department of Pharmacology and Toxicology, University of Alabama at Birmingham, 1670 University Boulevard, Volker Hall L106, Birmingham AL 35294, E-mail: Coral@uab.edu Phone: (205) 934-7139; Fax: (205) 934-8240

Abstract:

Bisphenol A (BPA) is a synthetically made chemical used in the production of polycarbonate plastics and epoxy resins. Recent studies have shown over ninety percent of humans investigated have detectable BPA concentrations. Yet, the biggest concern for BPA is exposure during early development because BPA has been shown to bind to the estrogen receptors (ER) and cause developmental and reproductive toxicity. We have investigated the potential of perinatal BPA to alter susceptibility for chemically-induced mammary cancer in rats. We demonstrate that prepubertal exposure to low concentrations of orally administered BPA given to lactating dams resulted in a significantly decreased tumor latency and increased tumor multiplicity in the dimethylbenz[a]anthracene (DMBA) model of rodent mammary carcinogenesis. Our data suggested that the mechanism of action behind this carcinogenic response was mediated through increased cell proliferation, decreased apoptosis, and centered on an up-regulation of steroid receptor coactivators (SRCs) 1-3, erbB3, and increased Akt signaling in the mammary gland.

Also, we demonstrate that prenatal exposure to BPA shifts the time of susceptibility from 50 days to 100 days for chemically-induced mammary carcinogenesis. Proteomic data suggest that prenatal BPA exposure alters the expression of several proteins involved in regulating protein metabolism, signal transduction, developmental processes, and cell cycle and proliferation. Increases in ER-alpha, SRCs 1-3, Bcl-2, epidermal growth factor –receptor (EGFR), phospho-IGF-1R, phospho-c-Raf, phospho-ERKs 1/2, phospho-ErbB2 and phospho-Akt are accompanied by increase in cell proliferation. We conclude that exposure to low concentrations of BPA during the prenatal and early postnatal periods of life can predispose for chemically-induced mammary cancer.

Keywords: bisphenol A, mammary cancer, proteomics, cell proliferation, apoptosis

Introduction:

Steroid hormones play a prominent role in development. This extends from procreation to senescence. Timed expression and interactions of steroids, receptors and co-regulators help to determine differentiation, development, maturation and maintenance of organs and organelles. Even subtle structural modifications in steroid molecules can result in major biological differences that can be evidenced by alterations to gene and protein expressions.

Steroid receptors have specificity, but they can also bind similar structures, including some environmental chemicals. Aberrant activation of steroid receptors by environmental chemicals during early development can lead to immediate modification of biological signaling or even to long-term effects. Some of these changes are due to direct effects while the delayed and/or permanent alterations are hypothesized to be organizational effects (1, 2). These changes in protein/enzyme expression can lead to disease manifestations.

While chemical structure is important, dose is another factor to consider. Now, we realize that not all biological responses abide by a linear dose response, whereby a low dose elicits a lesser effect than higher doses (3). Indeed, different doses of 17ß-estradiol (E2) result in diverse outcomes for mammary tumors induced by dimethylbenz[a]anthracene (DMBA) in rats. Low doses have been reported to cause a marked stimulus in tumor growth, whereas much larger doses cause inhibition of tumor growth (4). Furthermore, environmental factors play a large role in cancer risk. These can be natural components of our foods or environmental chemical contaminates (5). A significant increase in cancer incidences was evidence shortly after the industrial revolution which is considered to have started in the 17th century. And it became even more frequent in the beginning of the 20th century. This may be associated with the production of environmental chemicals which can be direct acting carcinogens or even to those that are hormone mimics.

One such chemical is bisphenol A (4,4’-dihydroxy-2,2-diphenylpropane, BPA). BPA has two phenol rings that play a role in making polycarbonate plastic and epoxy resins. Polycarbonate plastics are found in the preparation of infant formula and water bottles, children’s toys, sport equipment, medical and dental devices, CDs, DVDs and household electronics. Epoxy resins of BPA are used in coatings in food and beverage cans. It is found in carbonless copy sale receipts and thermal papers. Global production of BPA was estimated to be more than 2.2 million tons in 2009. The primary route of exposure to humans occurs through the oral route, due to the leaching of BPA from incomplete polymerization of epoxy resins or degradation of the weak ester bonds that link the BPA monomers. Studies have shown that these bonds are frequently hydrolyzed during normal use, with factors such as time, elevated temperature, and pH extremes accelerating this process (6-9). Detectable concentrations of BPA have been found to leach from canned fruits, vegetables, and meat products, condensed and infant milk, canned sodas and juice, cardboard milk and juice containers, plastic food wrap, hospital intravenous tubing, and

polycarbonate food and beverage containers under extreme as well as normal conditions of use (8-11).

BPA has been routinely detected in human biofluids and tissues. In a 2005 study, Calafat et al. found 95% of adults surveyed had detectable concentrations of total (free + conjugated) urinary BPA (12). A pilot study measuring the urinary concentration of a panel of environmental chemicals reported a similar proportion (90%) of girls with detectable concentrations of urinary BPA metabolites (13). Total BPA concentrations ranged from 0.3 µg BPA/L to 54.3 µg BPA/L and averaged 2.0 µg BPA/L (3.0 µg BPA/g creatinine). A recent large scale study involving over 2,000 participants supported the findings of both studies (14). They reported the average concentration of 2.6 µg BPA/L. Conservative estimates based on the values provided in these and other studies suggest that most adults are exposed to approximately 0.05-1 µg BPA/kg body weight (BW) per day, while the high-end of a biologically achievable exposure does not likely exceed 9-10 µg BPA/kg BW per day (15-18).

Once ingested, BPA is absorbed through the gastrointestinal tract and transported, via the venous circulation, to the liver. First pass metabolism results in the induction of Phase II enzymes and the subsequent conjugation of the majority of BPA absorbed (19, 20). In rodents, non-human primates, and humans, uridine diphosphate glucuronosyl transferase is reported to produce the major metabolite of BPA, BPA-glucuronide (19-21). Most studies agree that the conjugates are biologically inert and thus all downstream effects are generally attributed to the action of the remaining free, unconjugated BPA (22). Several studies have been published chronicling the pharmacodynamics of BPA in rodent models. The elimination of BPA in these studies occurs quickly, with the majority of the administered dose being eliminated within 24 hours (19, 20). Volkel et al. found that adult humans were capable of clearing a single, orally administered bolus (5 mg BPA/person or 54-94 µg BPA/kg BW) within 24 hours (20). The half-life was recorded as 5.3 hours.

This emphasis on the route of administration becomes important amid recent criticism by industry and regulatory agencies that many of the studies designed to evaluate the health hazards posed by BPA use artificial routes of exposure that bypass first pass metabolism and thus subject the animal to much higher concentrations of parental BPA (19). This criticism is not without merit. It has become increasingly apparently that orally administered BPA is subjected to first pass metabolism and undergoes rapid elimination from the body after a single dose (19-21). Other methods of administration, such as intraperitoneal or subcutaneous injections, have been shown to produce increased bioavailability, decreased time to maximum concentration, increased maximum concentration, and a difference in the metabolites produced (19).

As with estrogen (3-5), dose is also an important consideration in BPA research. The current regulations on daily BPA exposure in the United States are largely based on a study conducted by the National Toxicology Program (23). In order to define clear limits of toxicity, F433 rats were fed BPA over a two-year period, resulting in the lowest-observed-adverse-effect level (LOAEL) of 50 mg BPA/kg BW per day (23). The US

Environmental Protection Agency (US EPA) applied a 1000-fold "safety factor" to this concentration to calculate a daily tolerable reference dose of 50 µg BPA/kg BW per day. It should be noted that despite this dose being significantly lower than the reported LOAEL, it still represents an exposure to BPA that is estimated to be at least five-fold greater than an exposure that can be realistically achieved through dietary intake in humans.

In the past few decades, much effort has gone into estimating daily human intake of BPA. Several estimates based on patterns of normal dietary consumption and BPA migration values currently exist. The NTP-CERHR recently reviewed this data, estimating that general population adults were exposed to 0.008-1.5 µg BPA/kg BW per day (24). The European Union estimated that most adults were exposed, at most, to 1.4 µg BPA/kg BW per day through food sources alone (25). Consuming large quantities of wines produced in vats lined with epoxy resins was estimated to result in a maximum exposure of 7.5 µg BPA/kg BW per day (25). Combined, this produced a maximum worst case scenario of normal human consumption of 9 µg BPA/kg BW per day and led to the maximum tolerated dose of 10 µg BPA/kg BW per day.

By most accounts, the deleterious actions of BPA stem from its weak ability to bind with the estrogen receptors (ERs) and induce transcription of estrogen response elements (EREs). This has been shown by multiple groups through a variety of in vitro modeling systems (22, 26). Several groups have shown the ability of BPA to compete with E2 for binding to the ERs, albeit at an affinity reported to be 2,000- to 10,000-fold less than E2 (26, 27). While it has been reported that BPA exhibits a greater affinity to ER-beta than ER-alpha and differences exist between the co-regulator proteins recruited to each of the ERs in the presence of BPA, none of these studies have shown that this translates to a greater ability of ER-beta to induce down-stream ERE-mediated gene transcription (22, 27).

While it is convenient to attribute the bulk of BPA's deleterious effects on its ability to function as a weak ER agonist (via AF-2 activation), it has also been shown to interact with the ERs in a manner that is entirely unique from all known classes of ER ligands (weak estrogens, pure agonists, and pure antagonists) (26). This suggests that perhaps the mechanism of action of BPA is much more complicated than originally thought.

Several studies have found *in vivo* effects of BPA related specifically to the mammary gland and the female reproductive tract. Fetal exposure to BPA in mice has been reported to reduce the age at time of vaginal opening, and reduce time between vaginal opening and first estrus (28). In rats, perinatal exposure to BPA disrupted estrous cyclicity and decreased serum luteinizing hormone in adulthood, suggesting involvement of negative feedback (29). Whether BPA or other xenobiotics impact preadolescents and onset of puberty or menarche is uncertain, given the paucity of longitudinal studies. Perinatal exposure to 250 ng BPA/kg BW per day through a subcutaneously implanted osmotic pump was observed to cause significant alterations in the mammary gland, including an increased number of terminal end buds (TEBs), a decreased rate of apoptosis in the TEBs, increased percentage of cells expressing the progesterone receptor (PR) in the mammary gland, and increased lateral branching (30). With gestational exposure alone,

BPA has been reported to increase the number of terminal ducts, TEBs, alveolar buds, and preneoplastic lesions in the mammary gland. Durando et al. have shown that prenatal exposure to BPA (via subcutaneously implanted osmotic pump) coupled to a sub-carcinogenic dose of N-nitroso-N methylurea (NMU) resulted in an increased percentage of preneoplastic and neoplastic lesions in the mammary gland (31). Recently, Murray et al. reported that fetal exposure to BPA induces mammary gland ductal hyperplasia and carcinoma in situ (32). Gestational exposure to BPA has been reported to result in reproductive and endocrine disruption in male and female rodents (33).

To investigate the potential of BPA to cause developmental toxicity and predisposition for mammary cancer, we first utilized a protocol whereby BPA exposure occurred during the early postnatal period. Since the primary route of exposure to BPA is oral, we administered BPA by gavage to lactating Sprague Dawley CD rats. We administered BPA on a daily basis to dams from day two postpartum until time of weaning on day 21 (34). We selected two BPA doses, a high dose given to the lactating dams that would not result in a change in body weight to the offspring and a second BPA dose that was one-tenth of the high dose (250 µg and 25 µg BPA/kg BW, respectively). Controls were treated with an equivalent volume of the vehicle, sesame oil, on the same schedule. In regard to potential developmental and endocrine toxicity, there were no significant alteration on body weight, puberty as assessed by vaginal opening, and circulating E2 and progesterone concentrations in 50 day old female rats (34).

For investigating susceptibility for chemically induced mammary cancer, we used the established DMBA-induced model. At day 50 postpartum, female offspring exposed prepubertally to 0, 25 and 250 µg BPA/kg BW were treated orally with 30 mg DMBA/kg BW. Day 50 in Sprague Dawley rats is routinely used for chemically-induced mammary cancer because this is a time of high mitotic index in mammary terminal end buds (35). Rats were subsequently palpated for mammary tumors, and necropsy was carried out at 180 days post DMBA exposure. As seen in Figure 1, prepubertal BPA exposure to rats resulted in a dose dependent increase in DMBA induced mammary tumors, with the high dose causing a significant increase in the number of tumors developing per rat. Furthermore, latency (time to first palpated tumor) was significantly decreased for BPA compared to sesame oil exposure. These results demonstrate that prepubertal only exposure to BPA can result in later increased susceptibility to chemically-induced mammary cancer in rats.

Using western blot analysis, steroid receptor co-activators (SRCs) 1-3, Akt, phospho-Akt, PR-A, and erbB3 proteins were determined to be significantly up-regulated at 50 days (34). We subsequently measured cell proliferation and apoptosis using the protein expression of Ki-67 and the TUNEL assay, respectively. At day 21, shortly after the last BPA treatment, we found no significant effect of prepubertal BPA exposure on cell proliferation and apoptosis in mammary glands of these rats. However at day 50, rate of cell proliferation was significantly increased and rate of apoptosis was significantly decreased in mammary glands of rats exposed to the high BPA dose compared to controls (Figure 2). Furthermore, the cell-proliferation-to-apoptosis ratio was over two-fold greater in the mammary glands of rats exposed prepubertally to BPA at 50 days of age

(34). Since the effects on cell proliferation and apoptosis were seen at day 50, and not at day 21 (shortly after BPA treatment), we surmise that these results were not due to direct BPA action, but rather to a "permanent" developmental effect, perhaps via organizational or imprinting mechanisms (1, 2, 36).

Extending our BPA studies to prenatal exposure, we treated pregnant Sprague Dawley rats with 0, 25 and 250 µg BPA/kg BW on days 2-20 postconception. In this manner, the fetuses were exposed transplacentally. At day 50 postpartum, female offspring were gavaged with 30 mg DMBA/kg BW to investigate chemically-induced mammary cancer. Interestingly, we found no difference between treated groups for mammary tumor multiplicity, latency or tumor incidence (37). Since we had previously investigated gene (38) and protein expressions (39) in mammary glands of rats exposed prenatally to BPA and found a greater number of significant changes at day 100 compared to day 50, we followed this by carrying out protein measurements at these ages.

Discovery proteomic studies were carried out *via* two-dimensional gel electrophoresis for protein separation and enrichment and mass spectrometry for identification. We used western blot analysis from a separate set of identically treated animals for protein validation. What became evident from our proteomic studies was that there were many proteins involved in regulating protein metabolism, signal transduction, developmental processes and cell cycle and proliferation (39) (Table 1). Hence, we elected to investigate low-abundance down-stream signaling proteins in mammary glands of 50 and 100 old females in order to determine the long lasting effects of prenatal exposure to BPA. Figure 3 demonstrates that at day 50, ER-alpha, PR-A, and Bcl-2 were down-regulated and only SRC-3 was up-regulated (39). At 100 days, ER-alpha, Bcl-2 and the SRCs 1-3 were up-regulated in mammary glands of rats prenatally exposed to BPA.

Probing further, we found that phospho-ERK-1 and 2, phospho-ErbB2 and phospho-Akt were up regulated in mammary glands of 50 day old rats prenatally to BPA (Figure 4). On the other hand, in mammary glands of 100 day old rats, epidermal growth factor receptor (EGFR), phospho-IGF-1 receptor, phospho-c-Raf, phospho-ERK-1 and 2, phospho-ErbB2 and phospho-Akt were up-regulated. Together, 11 of 12 proteins associated with cell proliferation were up-regulated in mammary glands of 100 day old rats and only five proteins that can be implicated with cell proliferation were up-regulated in mammary glands of 50 day old rats. In addition, we measured Ki-67 in the mammary epithelial cells of 100 day old rats and found a 2.25 fold increase in cell proliferation in prenatal BPA exposed rats compared to sesame oil exposed rats (31.14% and 13.84%, respectively) (37). This suggested to us that prenatal exposure to BPA may shift the timing of susceptibility for mammary cancer in rats.

Accordingly, we investigated DMBA-induced mammary cancer in 100 day old female rats whose dams were treated orally during pregnancy with 250 µg BPA/kg BW or the vehicle, sesame oil. In contrast to the results of rats exposed on day 50 with DMBA, we recorded a significant increase in tumor incidence, a nonsignificant increase in tumor multiplicity, and a significant decrease in time to first tumor development in 100 day old rats exposed prenatally to BPA (Figure 5) (37). Furthermore, the pathology report

revealed significantly increased proportion of 100 day old DMBA-induced mammary tumors classified as grade II according to the Bloom-Richardson system which takes into consideration mitotic index, nuclear grade and adenocarcinoma tubular pattern (40) in rats exposed prenatally to BPA (45%) as compared to sesame oil (23%). This suggested that prenatal BPA exposed offspring could develop more aggressive mammary cancer.

In summary, we have shown that prepubertal exposure to oral low concentrations of BPA resulted in a significantly decreased time to first tumor latency and increased tumor multiplicity in the DMBA model of rodent mammary carcinogenesis (34). Our data suggested that the mechanism of action behind this carcinogenic response was mediated through increased cell proliferation, decreased apoptosis, and centered on an up-regulation of SRCs 1-3, erbB3, and increased Akt signaling in the mammary gland.

Furthermore, we demonstrate that prenatal exposure to BPA shifts the time of susceptibility from 50 days to 100 days for chemically-induced mammary carcinogenesis (37). Proteomic studies prove valuable in elucidating mechanism of action (39). Increases in ER-alpha, SRCs 1-3, Bcl-2, EGFR, phospho-IGF-1R, phospho-c-Raf, phospho-ERKs 1/2, phospho-ErbB2 and phospho-Akt are accompanied by increase in cell proliferation (37).

Outlook:

Future research should investigate if prenatal and prepubertal exposures to orally administered BPA exert its long lasting effects via epigenetic mechanisms, and if populations at risk (certain phenotypes) are more likely to develop breast cancer if exposed to BPA. Finally, dose response studies (especially at low doses) and measurement of blood and urine BPA concentrations should be carried out in order to draw comparison to human exposure.

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Figure Legends:

Figure 1: Tumor multiplicity and latency of DMBA induced mammary tumors in rats exposed prepubertally to bisphenol A. Lactating dams were gavaged with 0, 25, or 250 µg BPA/kg BW per day from days two through 20 postpartum. There were 32, 34, and 24 female offspring in the SO, 25 BPA, and 250 BPA groups, respectively, all derived from individual litters. At day 50, all female offspring were gavaged with a single dose of 30 mg DMBA/kg BW. For multiplicity, values are provided as mean ± SEM of tumors per rat. Latency values indicate the median time to first palpable tumor, given in days. P values greater than or equal to 0.05 were considered significant. Adapted from ref 34.

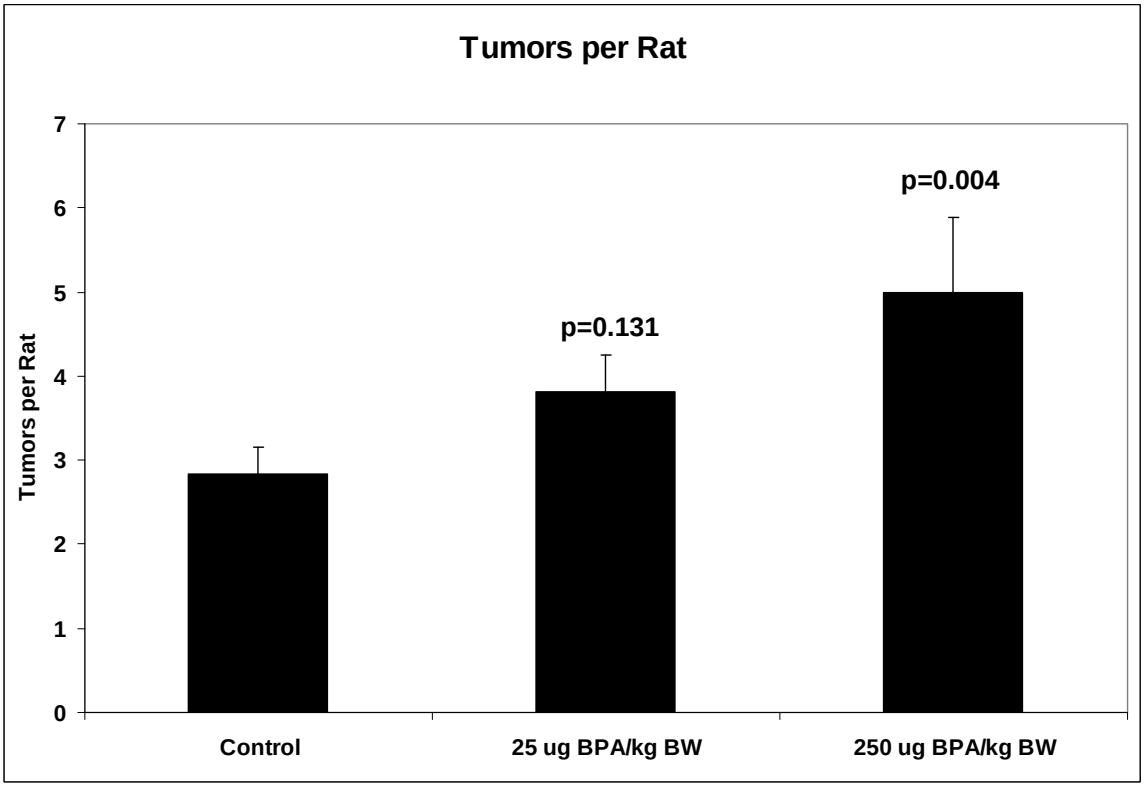
Figure 2: Cell proliferation and apoptosis in mammary glands of 50 day old rats exposed lactationally to dams treated with 250 µg bisphenol A (BPA)/kg BW per day. The graph illustrates mean index values ± SEM as a percent of the control group. The resulting numbers were used to construct a contingency table. Adapted from ref. 34.

Figure 3: Western blot analysis of ER-alpha, PR-A, Bcl-2, SRC-1, SRC-2, and SRC-3 in mammary glands of (*A*) 50-day-old and (*B*) 100-day-old rats exposed prenatally to 250 µg BPA/kg BW or an equal volume of sesame oil (controls). Values represent mean density ± SE as a percentage of the control, with densitometry values for controls set to 100; $n = 6–8$ samples per group. Insets are representative immunoblots for each protein per treatment. $*p < 0.05$ compared with corresponding controls. Adapted from reference 37.

Figure 4: Western blot analysis of EGFR, phospho-IGF-1R, phospho-c-Raf, phospho-ERK 1/2, phospho-ErbB2, and phospho-Akt in mammary glands of (*A*) 50-day-old and (*B*) 100-day-old rats exposed prenatally to 250 µg BPA/kg BW or an equal volume of sesame oil (controls). Values represent mean density ± SE as a percentage of the control, with densitometry values for controls set to 100; $n = 6–8$ samples per group. Insets are representative immunoblots for each protein per treatment. $*p < 0.05$ compared with corresponding controls. Adapted from reference 37.

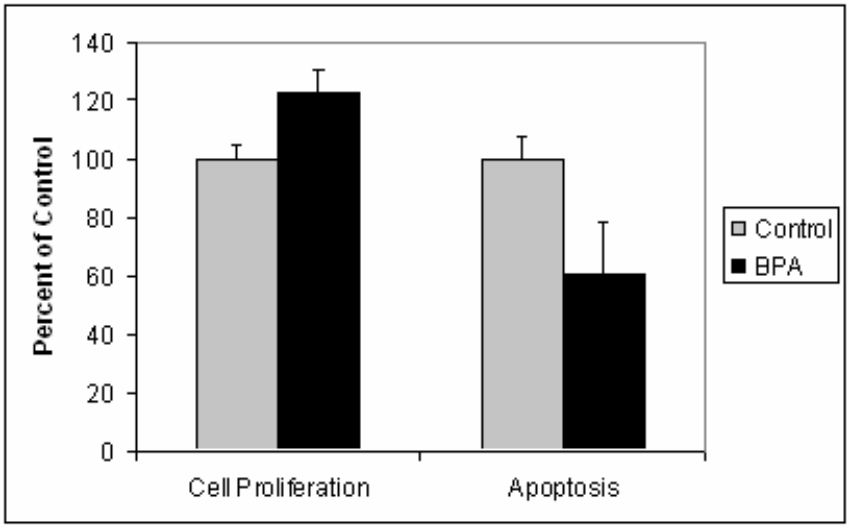
Figure 5: (A) Tumor multiplicity, (B) tumor incidence, (C) palpable tumor latency, and (D) tumor grade in female offspring prenatally exposure to 250 µg BPA/kg BW or an equal volume of sesame oil (controls) and gavaged with a single dose of 30 mg DMBA/kg BW on postnatal day 100. Adapted from reference 37.

Figure 1:



Treatment	Median Tumor Latency (days)
Control	65
25 µg BPA	56.6 (p=0.058)
250 µg BPA	53 (p=0.025)

Figure 2:



Proliferation			
Treatment (n)	Stained	Not Stained	p-value
Control (5)	432 (13.00%)	2890	<0.001
BPA (5)	526 (16.98%)	2572	
Apoptosis			
Treatment (n)	Stained	Not Stained	p-value
Control (5)	107 (2.52%)	4136	0.001
BPA (5)	95 (1.60%)	5851	

Figure 3:

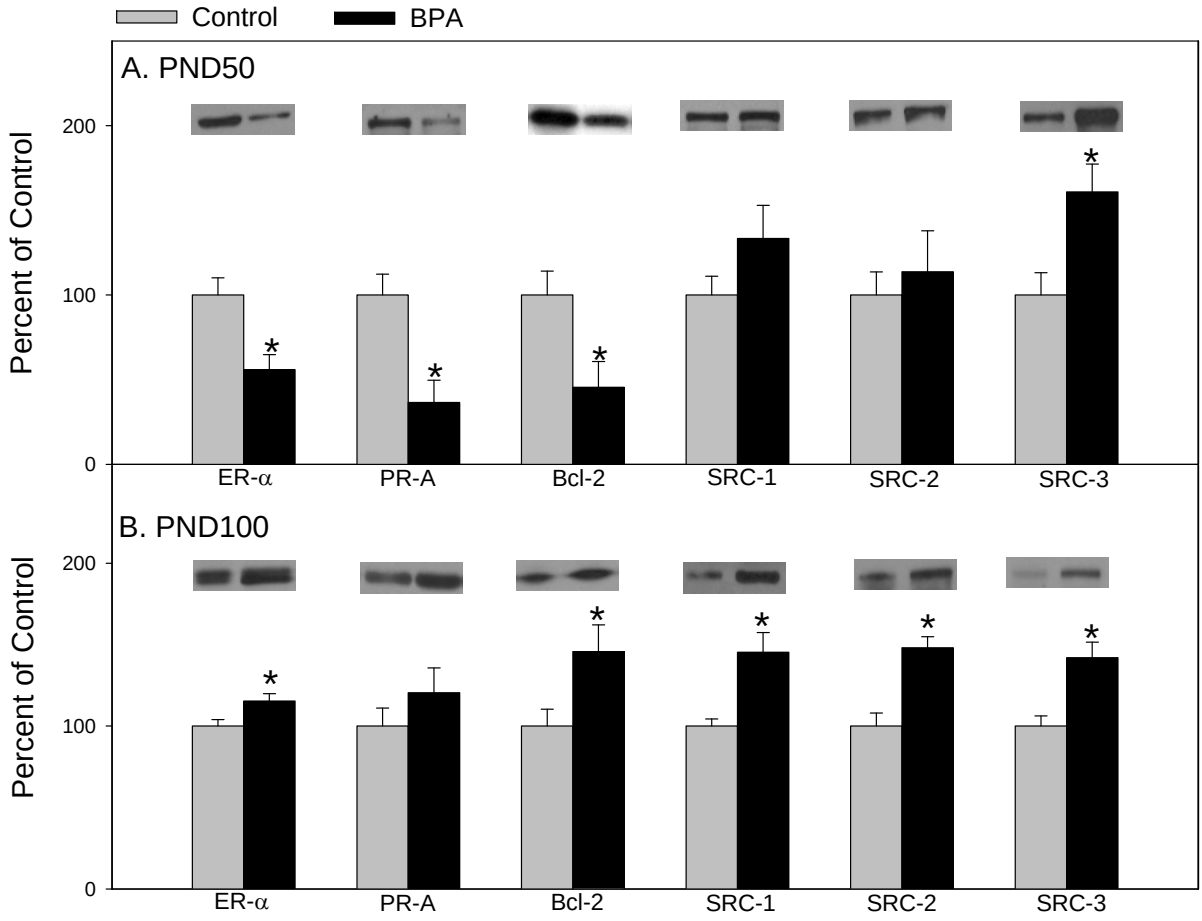

Control
BPA
A. PND50
Percent of Control
200
100
0
ER-α
PR-A
Bcl-2
SRC-1
SRC-2
SRC-3
B. PND100
Percent of Control
200
100
0
ER-α
PR-A
Bcl-2
SRC-1
SRC-2
SRC-3

Figure 4:

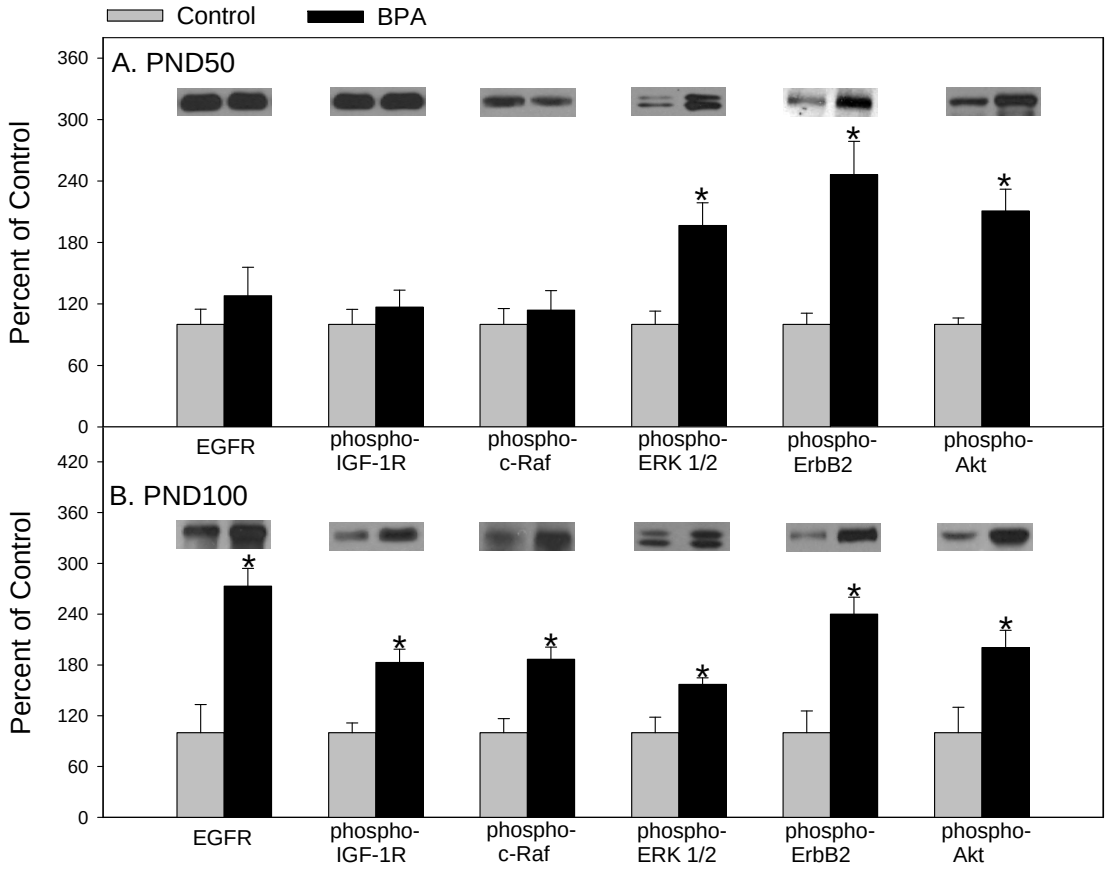
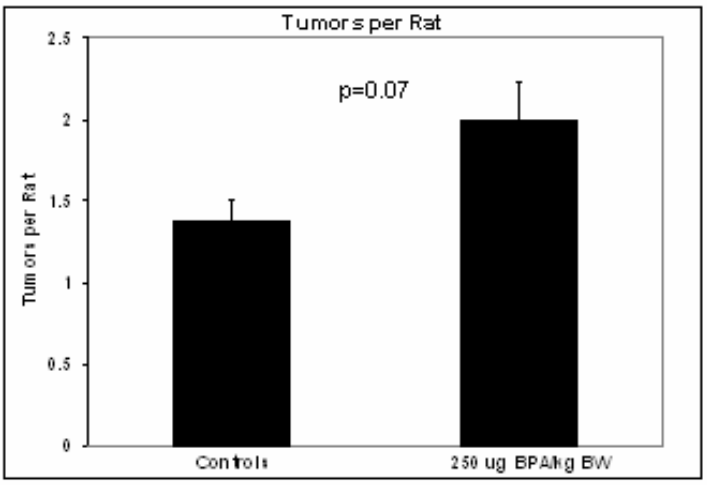

Control
BPA
A. PND50
Percent of Control
360
300
240
180
120
60
0
EGFR
phospho-IGF-1R
phospho-c-Raf
phospho-ERK 1/2
phospho-ErbB2
phospho-Akt
420
B. PND100
Percent of Control
360
300
240
180
120
60
0
EGFR
phospho-IGF-1R
phospho-c-Raf
phospho-ERK 1/2
phospho-ErbB2
phospho-Akt

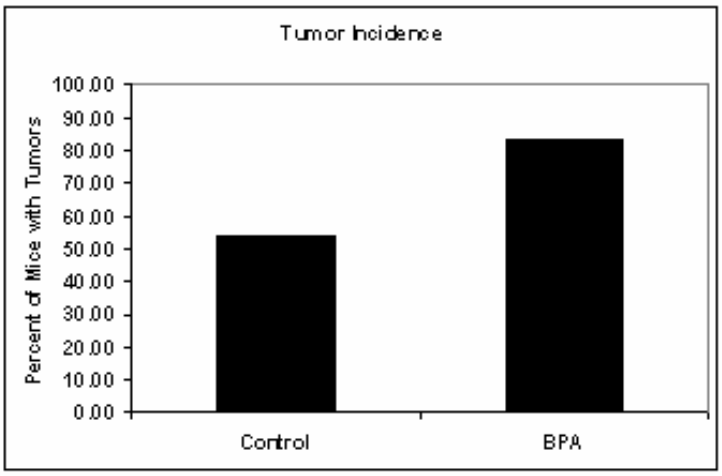
Figure 5:

Mammary Tumorigenesis in Rats Exposed Prenatally to BPA and at Day 100 to DMBA

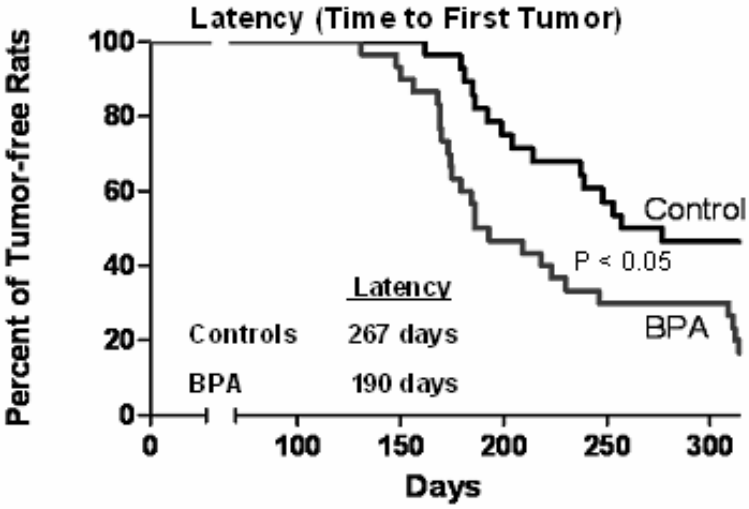
A.



B.



C.



D.

Tumor Grade

Prenatal – Day 100	0	I	II	% Grade II*
Control – DMBA	14	10	3	23%
BPA – DMBA	10	11	9	45% p < 0.05

*Bloom-Richardson grading scale (Modern Path 18:1067)

Table 1. Differentially expressed proteins as identified by mass spectrometry in mammary glands of rats exposed prenatally to BPA. Adapted from reference 39.

Protein Identification	Accession Number	Fold Change	Anova	BPA Treatment	Age	MW KDa	PI	Molecular Function
Aldose Reductase	P07943	-2.0	0.040	Low & High	50	36,230	6.3	Oxidoreductase
Tropomyosin beta chain	P58774	-1.6	0.018	Low	50	32,931	4.5	Cytoskeleton constituent
14-3-3 Protein epsilon	P62259	-1.6	0.032	Low	50	29,326	4.5	Protein domain specific binding
Alpha-1B-glycoprotein	P04217	-2.6	0.025	High	50	57,127	7.0	Immunoglobulin receptor
Heat shock cognate 71 kDa protein	P19378	1.3	0.035	High	50	70,989	5.1	Chaperone
Peroxiredoxin-2	Q61171	1.2	0.042	High	50	21,936	5.1	Oxidoreductase/ peroxidase
Fibrinogen gamma	Q8VCM7	-1.4	0.033	High	50	40,227	5.6	Protein binding
SPARC	P07214	-1.8	0.032	High	50	35,129	4.7	Extracellular matrix binding
SH3 domain-binding glutamic acid-rich-like protein 3	Q91VW3	-1.4	0.033	Low	50	10,527	4.9	Unclassified
ATP synthase subunit delta	Q9D3D9	-1.7	0.022	High	50	17,020	6.5	Hydrogen transporter
Actin, cytoplasmic 1	P60710	-1.5	0.043	High	100	42,052	5.2	Protein binding
Creatine kinase B type	Q04447	-3.1	0.042	Low & High	100	42,983	5.3	Kinase/ protein binding
Hemopexin Precursor	P02790	1.8	0.032	High	100	52,060	6.5	Iron ion binding
Tropomyosin alpha-3 chain	Q63610	-1.5	0.018	Low	100	29,217	4.6	Actin binding
Coronin 1A	O89053	-2.3	0.025	Low & High	100	51,026	6.2	Actin-binding protein
14-3-3 protein eta	P68510	1.9	0.012	Low	100	28,151	4.7	Protein domain specific binding
Vimentin	P20152	1.8	0.035	High	100	53,754	4.9	Structural protein/ Protein binding

APPENDIX B:

Jenkins S, , Betancourt AM, Wang J, Mobley JA, and Lamartiniere CA. Proteomic Basis for the Increased Susceptibility of the Mammary Gland to Carcinogenesis after Perinatal Exposure to Bisphenol A. Edited by Jose Russo. (**In Submission**)

Proteomic Basis for the Increased Susceptibility of the Mammary Gland to Carcinogenesis after Perinatal Exposure to Bisphenol A

Sarah Jenkins[1], Angela M. Betancourt[1], Jun Wang[1], James A. Mobley[1, 2, 3], and Coral A. Lamartiniere[1,3 *]

[1] Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL, USA

[2] UAB Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL, USA

[3] Division of Urology, Department of Surgery, University of Alabama at Birmingham, Birmingham, AL, USA

Corresponding Author: Coral Lamartiniere, 1670 University Blvd, Volker Hall L-106D, Birmingham, AL, 35294; coral@uab.edu

Outline

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- Subsection X.2.2: The Effects of Early Life Exposure to Bisphenol A *In Vivo*

Section X.3: Proteomics as a Method to Determine Complex and Novel Mechanisms of Action
- Subsection X.3.1: Introduction to Proteomics
- Subsection X.3.2: Two-Dimensional Gel Electrophoresis

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- Subsection X.4.1: Prepubertal Exposure to Bisphenol A
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Section X.6: Summary

Section X.1: Environmental Contaminants and Breast Cancer Development

Breast cancer etiology attributed to hereditary causes is low, only accounting for an estimated 5-15% of all diagnosed cases **[1]**. This is suggestive of other causative factors being involved. Extrinsic factors, such as the environment, diet, and lifestyle, therefore, may play substantial roles in the high incidence of breast cancer. The myriad of chemical contaminants present in the environment represent one potentially contributing factor. One specific group of chemicals, termed endocrine disruptors, are capable of altering endocrine signaling. Because normal endocrine signaling, especially that of the sex steroid hormones, has been shown to play a vital role in the development and progression of breast cancer, chemicals found to disrupt these signaling pathways may present the most obvious threat. For this reason, environmental contaminants with endocrine disrupting properties are currently being scrutinized for their potential role in breast cancer causation and progression.

One of the best known examples of an environmental endocrine disruptor influencing the development of human pathology is found in the case of the synthetic estrogen, diethylstilbestrol (DES). Women were prescribed DES from 1947 until 1971 to prevent spontaneous abortions. However, not only was the drug therapeutically ineffective in reducing the incidence of miscarriages, but research has revealed other, unintended side effects. Women exposed *in utero* to DES have shown an increased incidence of a rare type of vaginal cancer during adolescence **[2]**. As this population of women prenatally exposed to DES has aged, it has become increasingly apparent that they are also more susceptible to developing breast cancer than their unexposed, age-matched counterparts.

Indeed, a recent study found that DES-exposed women greater than 40 years of age exhibited a significant increase in the relative risk of developing breast cancer [3]. This increased susceptibility towards developing breast cancer was even more pronounced in prenatally DES-exposed women older than 50 years of age, though the small sample size limited statistical significance [3]. Importantly, these effects, occurring more than a decade after the original exposure, have caused greater public awareness of the role that early life exposure to endocrine disruptors can play in lifetime breast cancer risk. Likewise, recent media attention has focused on another common environmental contaminant, Bisphenol A (BPA), as a potentially harmful agent due to its classification as an endocrine disruptor and its widespread exposure to humans. This has led to extensive research into the potential adverse health effects elicited by early life BPA exposure and the molecular origin of these latent actions in the mammary gland.

Section X.2: Bisphenol A, the Widespread Environmental Contaminant

Subsection X.2.1: Human Exposure to Bisphenol A

BPA is a synthetically made chemical used in the production of polycarbonate plastics and epoxy resins. These products are used to manufacture a plethora of commonly used consumer goods, such as plastic food and beverage containers, the lacquer lining of canned foods and drinks, infant formula bottles, office water coolers, laboratory supplies, intravenous tubing, and some dental sealants, among other uses. The primary route of human exposure is believed to occur through the oral route due to the leaching of BPA from incomplete polymerization or polymer bond degradation. Detectable concentrations of BPA have been found to leach from canned fruits, vegetables, and meat products, condensed and infant formula milk, canned sodas and juice, infant formula bottles,

cardboard milk and juice containers, plastic food wrap, and polycarbonate plastic food and beverage **[4-13]**. As such, the scope of BPA exposure in the general public extends far beyond that of DES. Indeed, BPA has been routinely detected in human biofluids and tissues, including urine, serum, maternal and fetal plasma, amniotic fluid, breast milk and colostrum, mammary adipose tissue, and placental tissue **[14-16]**. In the United States, studies have consistently reported that greater than 90% of the populations under study have detectable concentrations of BPA metabolites in the urine, indicating widespread exposure **[17-19]**. However, it is the detection of BPA in biofluids and tissues accessible to the developing fetus and infant which present a special concern, as limited hepatic capacity for BPA metabolism exists in early life.

Subsection X.2.2: The Effects of Early Life Exposure to Bisphenol A *In Vivo*

A number of studies have linked early life exposure to low concentrations of BPA with a host of reproductive and developmental abnormalities in both sexes **[20]**. BPA has been shown to alter body weight, fat composition and distribution, estrous cyclicity, oogenesis, the timing of puberty, behavior (aggression, hyperactivity, learning, etc.), and circulating levels of lipids and leptin, among other effects **[20]**. Early exposure to BPA has been shown to increase the susceptibility of male rodents to prostate cancer, decrease serum testosterone concentrations, and increase prostate gland weights **[21-27]**. A number of effects have also been reported on metabolism, including alterations in blood glucose homeostasis and cellular glucose uptake **[28, 29]**.

Because the mammary gland is a complex organ whose development spans from *in utero* to adulthood, it was hypothesized that early life exposure to BPA may alter normal

patterns of development or induce pathology. Indeed, several studies have found effects of BPA related specifically to the mammary gland. Perinatal exposure to 250 ng BPA/kg body weight (BW) per day through a subcutaneously implanted pump was observed to cause significant alterations in the mammary glands, including increased number of terminal end buds (TEBs), decreased rate of apoptosis in the TEBs, and increased percentage of cells expressing the progesterone receptor in the mammary gland, and increased lateral branching **[30]**. With gestational exposure alone, BPA has been reported to increase the number of terminal ducts, TEBs, alveolar buds, and preneoplastic lesions in the mammary gland **[31, 32]**. Further, Durando et al. have shown that prenatal exposure to BPA (via subcutaneously implanted osmotic pumps) coupled to a sub-carcinogenic dose of N-nitroso-N-methylurea (NMU) resulted in an increased percentage of preneoplastic lesions in the mammary gland **[33]**. However, in spite of this, the actual molecular actions of BPA that initiate these alterations remain largely unknown.

Section X.3: Proteomics as a Method to Determine Complex and Novel Mechanisms of Action

Subsection X.3.1: Introduction to Proteomics

The use of traditional laboratory techniques, such as immunoblotting and immunohistochemical staining, in attempts to elucidate novel pathways altered by pathological states or chemical exposure can largely result in unsuccessful guesswork. This approach tends to rely solely on logical, familiar, and previously identified pathways, thus limiting its usefulness in deciphering novel pathways of regulation. The use of relatively new, high throughput technologies, such as genomic and proteomic screening tools, have become attractive alternatives. Broadly, these tools allow one to

determine global changes in mRNA or protein expression for use in hypothesis generating research. While genomic tools provide a more developed, standardized methodology, it is undercut by the lack of consistency in mRNA expression predicting protein expression. For this reason, we have recently explored proteomic technology.

Subsection X.3.2: Two-Dimensional Gel Electrophoresis

Since its advent in the 1970s, two-dimensional (2D) gel electrophoresis has been one of the most utilized methods for multidimensional protein fractionation. 2D gel electrophoresis functions by harnessing the chemical and structural differences inherent in proteins, namely the isoelectric point and molecular weight. Beginning from a crude protein mixture derived from lysed cells or whole tissue, proteins are absorbed, passively or actively, into dehydrated immobilized pH gradient (IPG) strips. The first dimension, termed isoelectric focusing, causes the protein mixture to separate according to each individual protein's isoelectric point, the pH at which a protein becomes electrically neutral. Briefly, proteins not at their designated isoelectric point along the pH gradient are charged. The application of an electrical current to the IPG strips causes the charged proteins to migrate along the pH gradient until they arrive at their specific isoelectric point. The focused IPG strips are then coupled to polyacrylamide gels, and the proteins are separated in a second dimension according to molecular weight. The resultant gel is populated with distinct protein spots which are visualized using various stains, such as SyproRuby, Silver Stain, etc. Gel images are converted into digital images using laser scanners, and these gels are compared using computer software.

Software programs align and match protein spots between gels, making it possible to detect changes in protein expression and/or shifts in placement (due to degradation, post-translational modifications, etc.). Statistical analysis identifies putative spots of interest, which are manually excised and subjected to trypsin digestion. Utilizing mass spectrometry, these peptides are used for protein identification. Thus, coupling 2D gels with mass spectrometry allows for a robust and well developed method for the global analysis of a large subsection of the proteome. This method can be used to identify novel pathways of altered protein expression for a given set of cells or whole tissues. Indeed, we have previously used this method to successfully access changes in protein expression patterns that can serve as possible biomarkers. This has included identifying novel pathways altered by xenoestrogen exposure **[34, 35]**. For this reason, we utilized 2D gel electrophoresis and mass spectrometry in order to identify novel alterations in protein expression in the mammary gland caused by early life exposure to BPA.

Section X.4: The Case of Early Bisphenol A Exposure

Given the complexity of mammary gland development, we investigated whether early life exposure to BPA could alter mammary cancer susceptibility in the well-established model of mammary cancer, dimethylbenz(a)anthracene (DMBA)-induced carcinogenesis in Sprague Dawley rats **[36, 37]**. This modeling system provides an acceptable method of inducing mammary carcinogenesis in a model organism that closely mimics human mammary gland development **[38]**. Furthermore, in order to mimic human exposure, we chose to administer BPA through the oral route only since that is the primary route by which humans are exposed to BPA. We selected two discrete early life exposure schemes to investigate the effects of BPA on two potentially critical windows of development,

prenatal and early postnatal life. The following sections describe our efforts to determine the carcinogenic properties of early life BPA exposure in an *in vivo* model system and the application of proteomics and traditional immunologic lab techniques to aid in identifying novel protein alterations that result from these effects.

Subsection X.4.1: Prepubertal Exposure to Bisphenol A

We first examined the impact of BPA exposure during the prepubertal period, which for our purpose was defined as the time frame shortly following birth (two days postpartum) until weaning (21 days postpartum) **[37]**. Briefly, female Sprague Dawley rats were bred and allowed to give birth. Beginning on postpartum day two and continuing through postpartum day 20, the lactating dam of each litter was intragastrically gavaged with 0 or 250 µg BPA/kg body weight/day. This exposure protocol resulted in an indirect, oral exposure of the offspring to BPA through the dam's milk. At 21 days of age, the female offspring were weaned from the lactating dam, concluding the window of BPA exposure. It is worth emphasizing that while we continued to monitor these rats post-pubertally, they were exposed to BPA during the prepubertal (postpartum days 2-20) period only.

These animals were sacrificed at select time points in order to determine and differentiate between direct and latent effects of BPA exposure. For this reason, we selected time points of 21 days postpartum (immediately following BPA treatment) and 50 days postpartum (~4 weeks following BPA treatment). Interestingly, no direct effects were observed for the end points investigated, such as protein expression and indices of cell proliferation and apoptosis, in the mammary glands of 21 day old rats **[37]**. However, latent effects were observed. At 50 days postpartum, four weeks after discontinuing BPA

treatment, we observed a significantly increased proportion of cells undergoing cell proliferation in the mammary glands of rodents prepubertally exposed to BPA (**Figure X.1**) **[37]**. This was coupled to a significant decrease in the proportionof cells undergoing apoptosis in the mammary glands of rats prepubertally exposed to BPA (**Figure X.1**). When these two indices were combined to produce a cell proliferation-to-apoptosis ratio, prepubertal exposure to BPA resulted in a ratio that was two-fold greater than the ratio of control-treated rodents.

Using the traditional DMBA model of rodent mammary carcinogenesis, exposure to BPA during the prepubertal period followed by 30 mg DMBA/kg body weight at 50 days postpartum resulted in increased carcinogenesis during adulthood **[36]**. This caused a significant increase in the average number of tumors per rat, with prepubertal BPA exposure increasing the average number of tumors by nearly two-fold (**Figure X.2**). Latency (time-to-1st-tumor formation) was significantly reduced by prepubertal BPA treatment. These data provided the first evidence that maternal exposure to BPA during lactation (prepubertal period) decreased tumor latency and increased mammary tumor multiplicity in the female offspring **[37]**. We concluded that increased cell proliferation and decreased apoptosis indices at the time of DMBA administration play a vital role in BPA's mechanism of action.

Subsection X.4.2: Prenatal Exposure to Bisphenol A

The initial formation of the mammary gland begins in mid- to late-gestation. For this reason, we next investigated whether exposure to BPA during *in utero* development could alter later life mammary gland development and cancer susceptibility **[36]**. For this

series of experiments, timed pregnant Sprague Dawley rats were intragastrically gavaged with 0 or 250 µg BPA/kg body weight during mid- to late-gestation, from postconception days 10 through 21. In this manner, the developing offspring were exposed indirectly to BPA during *in utero* development. Following birth, the female offspring were evaluated for alterations in body weight, the onset of puberty, patterns of estrous cyclicity, and the circulating concentrations of estradiol-17β and progesterone **[36]**. No significant alterations in early or adult body weights were observed. Likewise, no significant differences were found in the onset of puberty, estrous cyclicity, or in the circulating concentrations of estradiol-17β or progesterone. When prenatal exposure to BPA was coupled to the traditional DMBA model of rodent mammary carcinogenesis (wherein DMBA is administered at 50 days of age), no significant effects on tumorigenesis were observed. There were no significant changes in tumor latency (median time to tumor onset), multiplicity (average number of tumors per rat), or the grade of the developing tumors **[36]**. However, because we had previously observed that prenatal exposure to BPA (in the absence of DMBA) resulted in significantly more altered gene expression in the mammary glands of 100 day old female rats than in 50 day old female rats, we investigated if this translated to differences in the susceptibility of the mammary gland to carcinogenesis on 100 days of age **[36, 39]**.

Female Sprague Dawley rats were prenatally exposed to BPA as previously described. However, instead of administering DMBA on 50 days of age as in the previous tumorigenesis studies described here, DMBA was administered (30 mg DMBA/kg body weight) at 100 days of age. The timeline to palpable tumor onset and multiplicity were recorded. Upon necropsy, tumors were excised to confirm incidence and paraffin blocked

for histopathological grading. When prenatal exposure to BPA was followed by DMBA administration at 100 days postpartum, mammary carcinogenesis was significantly increased **[36]**. This was unlike the lack of significant differences observed when prenatal BPA exposure was paired to the traditional model of chemically-induced carcinogenesis where DMBA was administered at 50 days of age. Tumor incidence was significantly increased from a 54% incidence in control-treated rats to an 83% increase for BPA-treated females (**Figure X.3A**) **[36]**. Prenatal BPA exposure caused a stark and significant reduction in tumor latency (time-to-1st tumor development), with BPA treatment accelerating the median time to development by 78 days (**Figure X.3B**). Furthermore, prenatal exposure to BPA significantly increased the grade of tumors and trended towards increased tumor multiplicity (**Figures X.3C and X.3D**). Thus, in a model of chemically-induced mammary carcinogenesis, prenatal exposure to BPA shifted the window of mammary cancer susceptibility and increased mammary carcinogenesis. While direct comparisons cannot be made between the results of the prenatal and prepubertal BPA tumorigenesis studies, it seems as if BPA exposure occurring during the prenatal window results in the greatest disturbances for mammary cancer susceptibility. For this reason, we selected the prenatal BPA exposure protocol for further evaluation into the molecular mechanisms responsible for increase mammary cancer susceptibility.

Subsection X.4.3: Proteomics

In order to gain insight into the molecular mechanisms responsible for prenatal BPA exposure predisposing rodents for chemically-induced mammary carcinogenesis, we initially used 2D gel electrophoresis and mass spectrometry to identify novel protein targets of BPA action. We focused our attention at 50 and 100 days of age because these

ages are the most relevant to our tumorigenesis studies involving prenatal BPA exposure in a model of chemically-induced mammary cancer. For these studies, whole mammary glands were homogenized and prepared for 2D gel electrophoresis. The resultant gels were scanned and analyzed as previously described (**Figure X.4**) [**34**]. Prenatal exposure to BPA resulted in the identification of 13 unique proteins being differentially regulated in the mammary gland (**Tables X.1 and X.2**).

Some of the proteins identified as being regulated by 2D gel electrophoresis, such as heat shock cognate 71 kDa protein, have been previously identified as potential targets of BPA **[40]**. However, the majority of the proteins identified were novel, previously unreported potential targets of BPA's actions**, [34]**. In order to solidify the robustness of this initial discovery technique, we performed immunoblotting for a subset of these proteins using a separate set of identically treated rodents. This provided an independent method of validating differentially regulated protein identifications. Our efforts were focused on proteins which were reported to play key roles in mammary gland development and/or cancer pathology and for which we were able to obtain commercially available antibodies. Two of the proteins identified by 2D gel electrophoresis and mass spectrometry that were found to be significantly different in abundance following prenatal exposure to BPA were vimentin and 14-3-3 protein eta. These were validated by immunoblotting as being differentially regulated. (**Figure X.5**) **[34]**.

Vimentin is a structural cytoskeletal protein which belongs to a family of intermediate filaments. In cancer, vimentin expression is associated with a dedifferentiated malignant phenotype, increased motility and invasiveness, drug resistance, and poor clinical

outcome **[41, 42]**. An important process involved in the development of breast cancer is the concomitant loss of epithelial phenotype while gaining a mesenchymal phenotype. During development, the cell's intermediate filament status changes from a keratin-rich network to a vimentin-rich network in the epitheial-to-mesenchymal trasition. At 100 days of age, we found significantly increased protein expression of vimentin. This change suggests the possibility of prenatal BPA inducing an epithelial-to-mesenchmal transition. This change is consistent with cancer development, a conjecture supported by vimentin expression being up-regulated in breast cancer cells **[43]**.

14-3-3 is a family of highly conserved protein forms (alpha, beta, delta, sigma, epsilon, eta, and zeta), expressed in all eukaryotic cells. These proteins play a crucial role in regulating multiple cellular processes, including the maintenance of cell cycle checkpoints and DNA repair, the onset of cell differentiation and senescence, and the coordination of cell adhesion and motility. For this reason, disruption of normal expression patterns of 14-3-3 could contribute to a pathological phenotype. Indeed, prenatal BPA exposure increased the expression of 14-3-3 eta in the mammary glands of 100 day old rats. This increase in protein expression suggests that the actions of 14-3-3, specifically 14-3-3 eta, could be playing a role in BPA causing increased susceptibility for mammary cancer.

Using the bioinformatics data performed on the collective group of differentially regulated proteins identified by 2D gel electrophoresis, we began targeted investigations into highlighted cellular signaling pathways which are known to play roles in the development of cancer, epithelial-to-mesenchymal transition, signal transduction, cell

proliferation, and cell motility. These types of proteins are historically absent from 2D gels because they are present in low abundance or are too hydrophobic for solubilization. These proteins include the growth factor receptors, epithelial growth factor receptor (EGFR), erbB2, and insulin-like growth factor 1 (IGF-1) receptor, and downstream proteins involved in the Akt and Ras signaling pathways (**Figure X.6**).

Via immunoblots, we began with the EGF receptor family of receptor tyrosine kinases as this pathway was identified as being highly connected to the proteins (including Vimentin and 14-3-3) which we found to be differentially regulated by 2D gel electrophoresis. The EGF receptor (erbB1/HER1) is one member of a closely related family of receptor tyrosine kinases which includes: erbB1 (EGFR/HER1), erbB3 (nu/HER2), erbB3 (HER3), and erbB4 (HER4). Ligand binding induces activation *via* the generation of homo- or hetero-dimers. In activated form, these proteins function as the initiating kinase in several different signaling cascades which control global cell activities such as DNA synthesis, cell proliferation, and apoptosis **[44, 45]**. For this reason, the over-expression of several of these family members has been associated with a number of cancers, especially breast cancer. Over-expression of EGFR and erbB2 occur in approximately 50% and 15-30% of all breast cancer cases, respectively, and are generally associated with an unfavorable outcome.

Another protein tyrosine kinase is the insulin-like growth factor 1 (IGF-1) receptor. The IGF-1 receptor has been reported to cross-talk with both EGF receptor (erbB1/HER1) and erbB2 (nu/HER2) **[46]**. It has been reported to play roles in the formation of cancer, the development of chemoresistance, insulin signaling, and the aging process **[reviewed in**

[46-51]. Specific to the mammary gland, the IGF-1 receptor has been shown to play important roles in mammary gland development, differentiation, and in the development of carcinogenesis **[reviewed in [52]**.

The Akt pathway represents one of the major signaling pathways modulated by the EGF receptor, erbB2, and IGF-1 receptor. These growth factor receptors function by phosphorylating, and thus activating, Akt. Phosphorylation of Akt is associated with a loss of cell adhesion, a decrease in cell-matrix adhesion, a loss of apico-basolateral cell polarization, and induction of cell motility **[53, 54]**. The Akt pathway is also capable of feeding into the Ras signaling pathway through the activation (phosphorylation) of c-Raf.

The Raf-1 protein functions in the mitogen-activated protein kinases (MAPK) signal transduction pathway as part of a protein kinase cascade, functioning downstream of the Ras family of membrane-associated GTPases **[55]**. The terminal result of this pathway causes the activation (phosphorylation) of the extracellular signal-regulated kinases (ERKs). Activated ERKs, ERK 1 and ERK2, are pleiotrophic effectors of cell physiology and play an important role in the control of gene expression involved in the cell cycle, apoptosis, differentiation, and migration **[34]**. They function by regulating the activities of several transcription factors which are involved in cell proliferation.

Investigating these signaling pathways at both 50 and 100 days postpartum following prenatal exposure to BPA, we found that phospho-erbB2, phospho-Akt, phospho-ERK1, and phospho-ERK 2 were up-regulated at both 50 and 100 days postpartum (**Figure X.6**) [**36**]. Further investigation showed that EGF receptor, phospho-c-Raf, and phospho-IGF-

1 receptor were up-regulated in mammary glands of 100, but not 50, day old rats prenatally exposed to BPA (**Figure X.6**) **[36]**. As noted above, all three of these proteins have been reported to play a role in cell proliferation and could account for the shift in susceptibility to chemically-induced mammary cancer. This illustrates the point that while downstream proteins, such as c-Raf, ERK1, and ERK2, were also up-regulated at 50 days of age, a greater number of growth factor receptors and downstream targets were up-regulated or phosphorylated at 100 days of age by prenatal exposure to BPA **[34, 36]**.

We also found that prenatal BPA exposure resulted in up-regulating the estrogen receptor alpha (ER α) at 100 days of age and down-regulated it at 50 days of age (**Figure X.6**) **[36]**. Since ER regulates the expression of several genes and corresponding proteins, including PR and Bcl-2, we investigated their expression. The expression of downstream targets of ER (PR-A and Bcl-2) in the mammary glands on postpartum days 50 and 100 followed a similar expression pattern to ER at each age (**Figure X.6**) [**36**]. This suggests that ER may play an active role in increased tumorigenesis. This becomes a concern because DMBA-induced mammary cancer in rats is initially estrogen-dependent and 50-80% of all cases of breast cancer in humans are initially ER-dependent. Greater concentrations of ER α at the age of carcinogen administration in this model could lead to an increased opportunity for carcinogenesis. The steroid receptor co-activators, which primarily function to mediate the transcriptional function of nuclear receptors, were all up-regulated at 100 days of age (**Figure X.6**) [**36**]. This is in contrast to 50 days of age, where only SRC-3 was significantly up-regulated. [**36**]. This demonstrates how the ontology of ER alpha and the sex steroid co-regulator expression changed and may explain the shift in the window of mammary cancer susceptibility.

Our results, using a combination of proteomics and traditional laboratory immuno-techniques, provide a plausible mechanism of action of how prenatal BPA exposure could alter signaling molecules in the mammary gland that in turn could contribute to an increased susceptibility for mammary cancer. In response to prenatal exposure to BPA, initial alterations in proteins such as vimentin and 14-3-3 could lead to alterations to cellular architecture and increased expression and/or activation of the EGF receptor, erbB2 and IGF-1 receptor. These receptors can then initiate the signaling cascade by activating several downstream proteins, such as c-Raf or Akt. In the case of c-Raf, increased activation could lead to increased phosphorylation of the mitogen-activated protein kinases, ERK1 and ERK 2. These activated kinases are then imported into the nucleus where they phosphorylate specific transcription factors involved in cell proliferation. Likewise, elevated activation of Akt has been seen in several types of human malignancy, including breast, ovarian, lung, and thyroid cancers.

Further, there is an intriguing link between the steroid co-regulators and growth factor signaling. While steroid receptor co-regulators have been reported to directly influence steroid receptor action, they have also been suggested to serve as transcription factors that can directly enhance steroid-mediated gene transcription by regulating growth factor signaling. Because we found increased expression and/or activation of several growth factor receptors, ERα, and all three members of the p160 steroid receptor co-regulator family, this could translate to an increased ability for SRC and growth factor receptor cross-talk. Future research is needed to confirm this conjecture. Taken together with our tumorigenesis data, these data suggests that while mild disturbances do exist at 50 days of

age after prenatal BPA exposure, it is not until 100 days of age that prenatal BPA exposure induces widespread alterations in the expression and activation of proteins known to play vital roles in carcinogenesis.

Section X.5: Serum Biomarkers of Xenoestrogen Exposure

Subsection: X.5.1: Introduction to Serum Biomarkers of Exposure

While proteomic technologies often allow researchers to identify novel mechanisms by which biological processes occur, additional applications include the growing need to identify disease-related biomarkers. Ideally, biomarkers of exposure and susceptibility should utilize non-invasive or minimally invasive sample sources and accurately and reproducibly identify susceptible/effected individuals through the use of a panel of differentially abundant molecules. As the data presented above suggests, early life exposure to BPA has the ability to increase one's susceptibility to mammary cancer later in life. Thus, it is our goal to establish a quantitative approach to biomarker discovery that is of sufficient sensitivity to detect BPA-induced alterations in the serum.

The nature of blood serum makes it poorly suited for the same type of sample preparation and separation platform used in assessing the mammary gland proteome. An extensive dynamic range present in the circulating serum necessitates a more thorough enrichment step. We have opted to use Seppro IgY spin columns, which depletes the top seven highest expression proteins from serum. While this does lessen the dynamic range of protein expression in serum, a large dynamic range still persists. The application of 2D gel electrophoresis combined with even the most sensitive of stains (ex. Sypro Ruby and Cy dyes) would not be adequate to fully explore the depth of the serum proteome. In

order to delve deeper into low abundance proteins, we have implemented a multifaceted approach based on peptide tagging with isobaric labels using Tandem Mass Tag (TMT) technology coupled with multidimensional protein identification (MudPIT) technology.

The TMT chemistry that we show here is a six-plex isobaric mass tagging system. Isobaric mass tagging enables stoichiometric labeling of complex protein extracts for global "relative" quantification and identification by tandem mass spectrometry. Each tandem mass tag has a unique mass and reports sample-specific abundance of a labeled peptide during MS/MS analysis. Each tag is composed of three elements: 1) a protein reactive group, 2) a mass normalizer group, and 3) a mass reporter. The protein reactive group, an amine-reactive NHS-ester group, provides high-efficiency amine-specific labeling of proteins and peptides. The mass normalizer differs from tag to tag; it functions as a balance to the mass reporter, ensuring that each of the mass tags in the six-plex set have the same overall mass. This method of multiplex peptide labeling allows for the comparison of six different samples by their differing signature mass peaks.

Subsection X.5.2: Preliminary Data and Future Directions

We have completed preliminary studies to establish rigorous parameters of use. Rat sera were pooled, and six 100 µl aliquots were depleted of the most abundant serologic proteins as described in the previous section. To test the limit of detection and dynamic range of our methods, purified bovine lactoferrin was added in duplicate (12, 60, and 300 ng/100 µg of depleted rat sera). Each sample was digested, labeled with a unique isobaric tag, and analyzed by 2D LC-ESI-MS/MS. An in-depth protocol is shown in **Figure X.7**. The resultant data were analyzed with SEQUEST and BioInquire's ProteoIQ software.

The *in silico* generated 2D view of the proteins identified after a 2D LC-ESI-MS run are shown in **Figure X.8A**. The reproducibility, limit of detection, and dynamic range were calculated based on the internal bovine lactoferrin controls. As seen in **Figure X.8B**, the 2D LC-ESI-MS/MS analysis of the TMT labeled samples yielded a concentration-dependent lactoferrin profile and the lowest concentration of 12 ng/100 µg sample was reproducibly detected.

Our preliminary studies using rat serum show that we were able to identify 3,032 peptides, 774 of which were unique peptides. There were 282 unique protein groups identified, and 126 proteins which had more than one unique peptide identified. The number of proteins with unique peptides carrying more than one quantifiable mass tag was 87. Collectively, these data validate this method to quantify serum proteins that may serve as biomarkers of chemical carcinogen exposure. Future work will focus on applying this technology to both rodents and humans in order to identify biomarkers of exposure using an array of environmental chemicals.

Section X.7: Summary

In summary, these studies demonstrate the ability of prenatal only exposure to BPA to alter protein expression in the mammary glands of postnatal rats. Through discovery proteomics we were able to identify novel putative targets of the latent detrimental effects of prenatal BPA exposure. These novel protein identifications allowed us to perform targeted analysis of downstream signaling transduction pathways that may have been otherwise ignored. While these are purely descriptive studies at this point and differential regulation does not necessarily imply impaired functionality, these proteins represent

novel findings that may provide new directions for determining how the deleterious actions of BPA are mediated. This demonstrates the value of proteomics in identifying novel protein signaling pathways altered in the mammary gland following early life exposure to environmental chemicals.

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FIGURE LEGENDS

Figure 1. Cell proliferation and apoptosis in mammary glands of 50 day old rats exposed lactationally to dams treated with 250 µg bisphenol A (BPA)/kg BW per day. The upper panel depicts Ki-67 expression as an indicator of cell proliferation and the TUNEL assay as measure of apoptosis. Terminal end buds from five biologically distinct samples (*n*=5) were analyzed per treatment. The graph illustrates mean index values ± SEM as a percent of the control group. The resulting numbers were used to construct a contingency table. All images were taken at 40x magnification. The scale bar represents 100 µm.

Figure 2: DMBA induced mammary tumors in rats exposed lactationally to bisphenol A. Lactating dams were gavaged with 0, 25, or 250 µg BPA/kg BW per day from days two through 20 postpartum (Monday-Friday only). There were 32, 34, and 24 female offspring in the SO, 25 BPA, and 250 BPA groups, respectively, all derived from individual litters. At day 50, all female offspring were gavaged with a single dose of 30 mg DMBA/kg BW. Tumor multiplicity data was analyzed with the GENMOD procedures in SAS using Poisson regression on the tumor appearance rates. P values are provided for mean number ± SEM of tumors per rat. P values greater than or equal to 0.05 were considered significant.

Figure 3: A) Tumor incidence (proportion of rats that developed at least one tumor), B) Tumor multiplicity (number of tumors per rat), and C) Kaplan-Meier survival curve with median time (days) to first tumor in female offspring prenatally exposed to 250 µg

BPA/kg BW or equal volume of sesame oil for controls. Rats were gavaged with a single dose of 30 mg DMBA/kg BW on PND100. Statistical analyses for tumorigenesis are provided in the Statistical Methods section.

Figure 4: Representative proteomic profile of a postnatal rat mammary gland after prenatal exposure to BPA. 2-DE was performed on 150 µg of protein using 18 cm isoelectric focusing strips with a pH range of 4-7 in the first dimension and SDS-PAGE (12.5%) in the second dimension. The gel was stained with SYPRO Ruby protein stain. Protein spots that were differentially regulated were analyzed with SameSpots software, and identified by either MALDI-TOF-TOF or LC-MS/MS analysis.

Figure 5: Western blot analysis of vimentin and 14-3-3 eta in the mammary glands of 100 days old rats exposed prenatally to 250 µg BPA/kg BW or an equal volume of sesame oil. Quantitation is reported as percentage of control. Densitometry values for controls were set to 100. Value represents mean density ± SEM as a percent of the control group. N = 6-8 per group. Statistical significance was defined as a p-value ≤ 0.05.

Figure 6: Western blot analysis of phospho-erbB2, phospho-Akt, phospho-ERKs 1/2, EGF receptor, phospho-IGF-1 receptor, phospho-c-Raf, ER alpha, PR-A, Bcl-2, SRC-1, SRC-2, and SRC-3 in mammary glands of 50 and 100 day old rats exposed prenatally to 250 µg BPA/kg BW or an equal volume of sesame oil (controls). Quantitation is reported as percentage change from control. Densitometry values for controls were set to 100. Values represent mean density ± SEM as a percent of the control group. n = 6-8 samples

per group. Asterisk (*) indicates statistically significant difference in detected protein abundance compared to corresponding controls ($p < 0.05$).

Figure 7: Illustrates the schematic of sample processing of both serum and tissue samples utilizing the TMT 6-plex labeling system.

Figure 8A: The reproducibility, limit of detection, and dynamic range was calculated based on the internal bovine lactabumin controls. The 2-D LC-ESI-MS/MS analysis of the TMT labeled samples yielded a concentration-dependent lactoferrin profile and the lowest concentration of 12 ng/100 ug sample was reproducibly detected.

Figure 8B: To test the limit of detection and dynamic range of 2-D LC-ESI-MS/MS, purified bovine lactoferrin was added in duplicate (12, 60, and 300 ng/100 ug of depleted rat sera). Each sample was digested, labeled with a unique isobaric tag (see Fig. 2), and analyzed by 2-D LC-ESI-MS/MS. Resulting data were analyzed with SEQUEST and BioInquire's ProteoIQ software. The *in-silico* generated 2D PAGE view of the proteins identified after a 2D LC-ESI-MS run.

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Table 1: List of differentially expressed proteins from mammary glands of 21 and 50 day old rats exposed prenatally to BPA and sesame oil as identified by MALDI-TOF/TOF analysis.

ID #	Protein Identification	Accession #	Mascot Score	Sequence Coverage[a]	Fold Change[b]	p-value	MW (kDa)[c]	Age	pI[d]	Molecular Function
1	Aldose reductase	P07943	148	43	-2.0	0.040	36	50	6.3	Oxidoreductase
2	Alpha 1B-glycoprotein	P04217	189	18	-2.6	0.025	57	50	7.0	Immunoglobulin receptor
3	Heat shock cognate 71 kDa protein	P19378	194	34	1.3	0.035	71	50	5.1	Chaperone
4	Peroxiredoxin-2	Q61171	184	35	1.2	0.042	22	50	5.1	Oxidoreductase/peroxidase
5	Actin, cytoplasmic 1	P60710	152	36	-1.5	0.043	42	100	5.2	Protein binding
6	Creatine kinase B type	Q04447	261	42	-3.1	0.042	43	100	6.5	Kinase/protein binding
7	Hemopexin Precursor	P02790	123	25	1.8	0.032	52	100	6.5	Iron ion binding

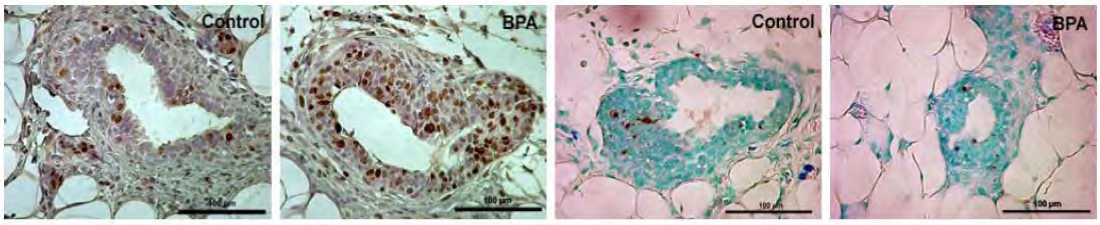
[a] Percent of identified sequence of the known protein.
[b] Positive and negative fold change in protein expression indicates up- and down-regulation of protein expression, respectively.
[c] Molecular weight of the matching protein in kDa.
[d] Isoelectric point of the matching protein.

Table 2: List of differentially expressed proteins from mammary glands of 21, 50, and 100 day old rats exposed prenatally to BPA and sesame oil as identified by LC-MS/MS analysis.

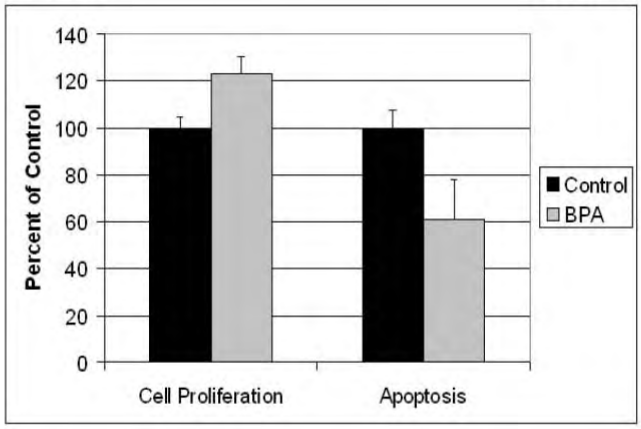
ID #	Protein Identification	Accession #	Xcorr Score	Matching Peptides[a]	Fold Change[b]	p-value	MW (kDa)[c]	Age	pI[d]	Molecular Function
8	Fibrinogen gamma	Q8VCM7	4.2	4	-1.4	0.033	40	50	5.6	Protein binding
9	SPARC	P07214	3.4	3	-1.8	0.032	35	50	4.7	Extracellular matrix binding
10	ATP synthase subunit delta	Q9D3D9	5.0	2	-1.7	0.022	17	50	6.5	Hydrogen transporter
11	Coronin 1A	O89053	3.29	2	-2.3	0.025	51	100	6.2	Actin-binding protein
12	14-3-3 eta	P68510	4.8	3	1.9	0.012	28	100	4.7	Protein domain specific binding
13	Vimentin	P20152	4.3	5	1.8	0.035	54	100	4.9	Structural protein/ protein binding

[a] Number of matching peptides.
[b] Positive and negative fold change in protein expression indicates up- and down-regulation of protein expression, respectively.
[c] Molecular weight of the matching protein in kDa.
[d] Isoelectric point of the matching protein.

Figure 1:



Ki-67 Staining TUNEL Assay



Proliferation			
Treatment (n)	**Stained**	**Not Stained**	**p-value**
Control (5)	432 (13.00%)	2890	<0.001
BPA (5)	526 (16.98%)	2572	
Apoptosis			
Treatment (n)	**Stained**	**Not Stained**	**p-value**
Control (5)	107 (2.52%)	4136	0.001
BPA (5)	95 (1.60%)	5851	

Figure 2:

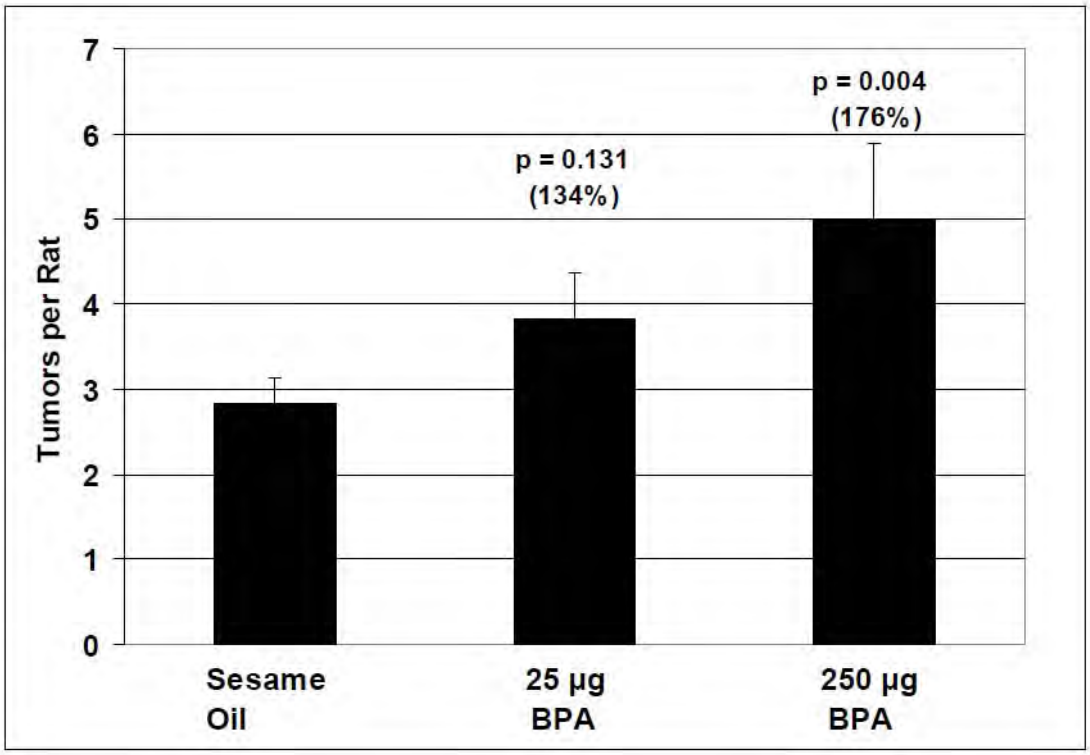
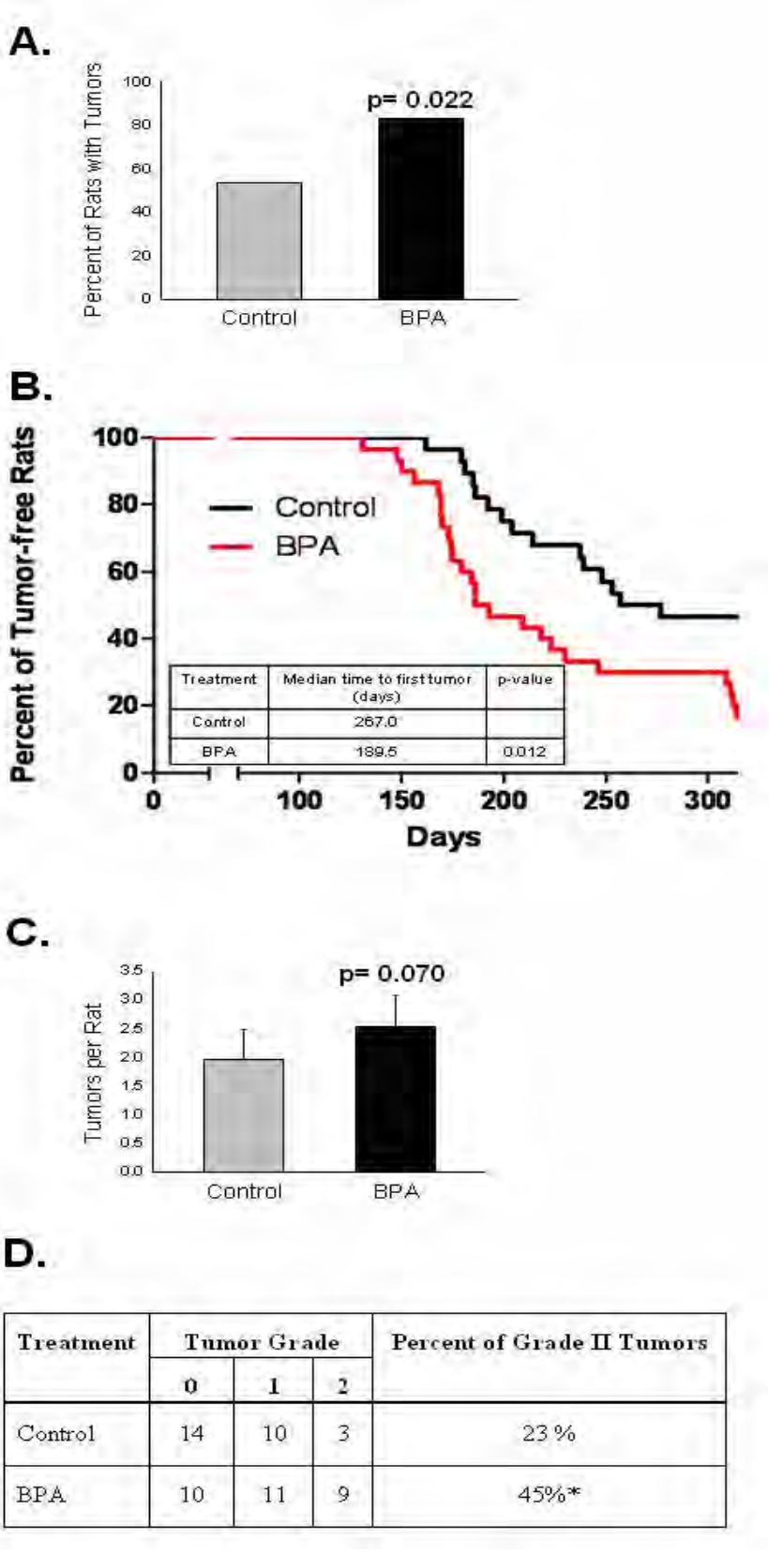

Tumors per Rat
7
6
5
4
3
2
1
0
p = 0.004
(176%)
p = 0.131
(134%)
Sesame Oil
25 µg BPA
250 µg BPA

Figure 3:



D.

Treatment	Tumor Grade			Percent of Grade II Tumors
	0	1	2	
Control	14	10	3	23 %
BPA	10	11	9	45%*

Figure 4:

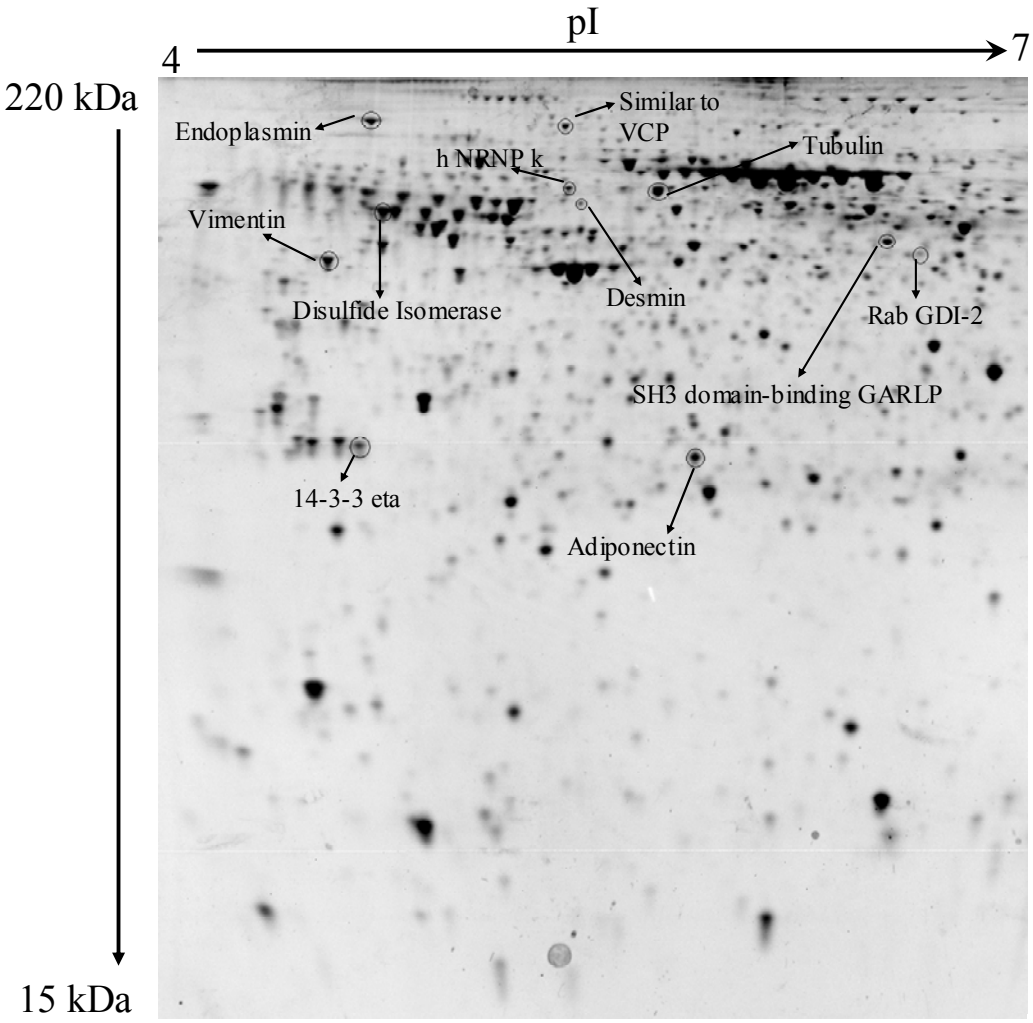

pI
4
7
220 kDa
15 kDa
Endoplasmin
Similar to VCP
h NRNP k
Tubulin
Vimentin
Disulfide Isomerase
Desmin
Rab GDI-2
SH3 domain-binding GARLP
14-3-3 eta
Adiponectin

Figure 5:

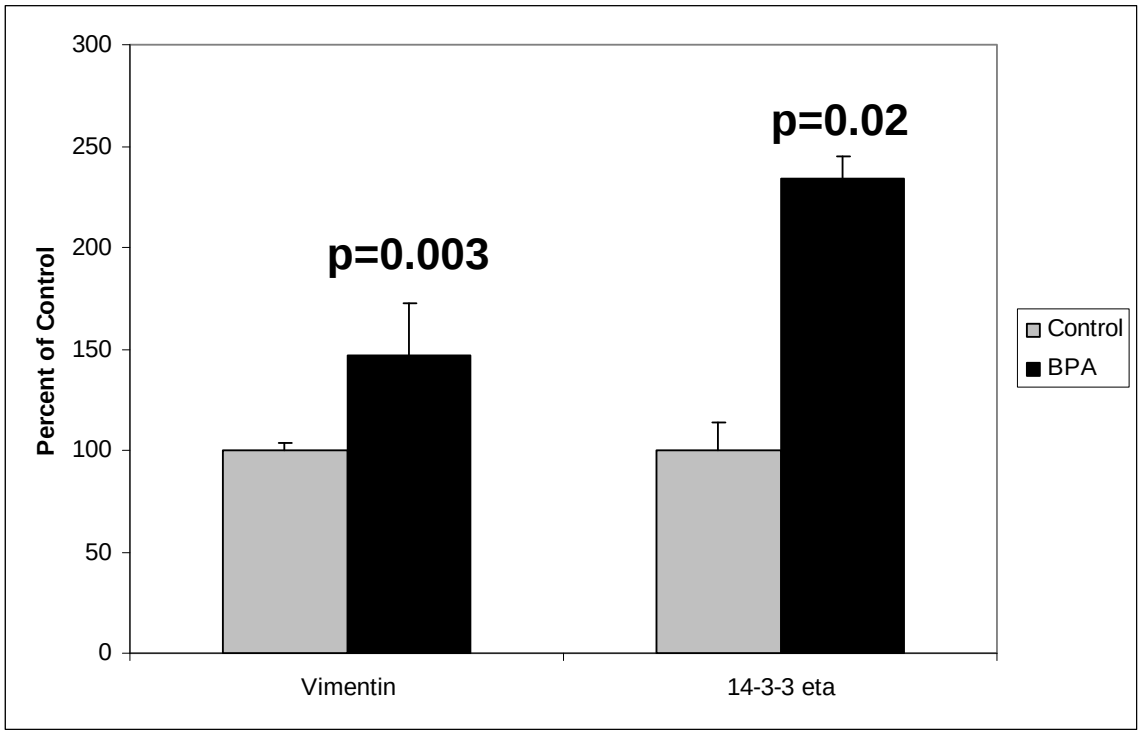
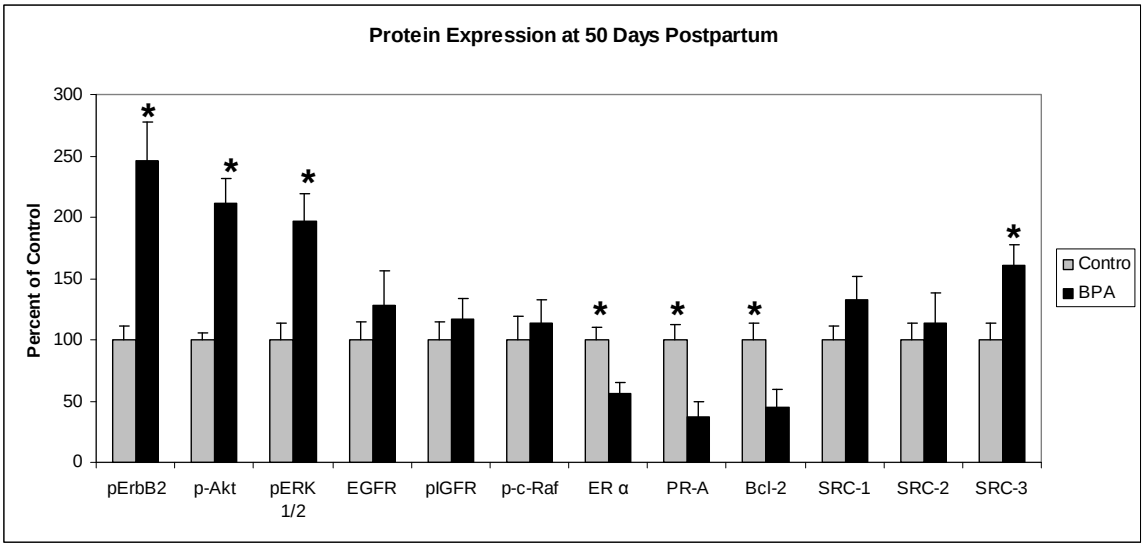

300
250
200
150
100
50
0
Percent of Control
p=0.003
p=0.02
Control
BPA
Vimentin
14-3-3 eta

Figure 6:


Protein Expression at 50 Days Postpartum
Percent of Control
300
250
200
150
100
50
0
pErbB2
p-Akt
pERK 1/2
EGFR
pIGFR
p-c-Raf
ER α
PR-A
Bcl-2
SRC-1
SRC-2
SRC-3
Contro
BPA

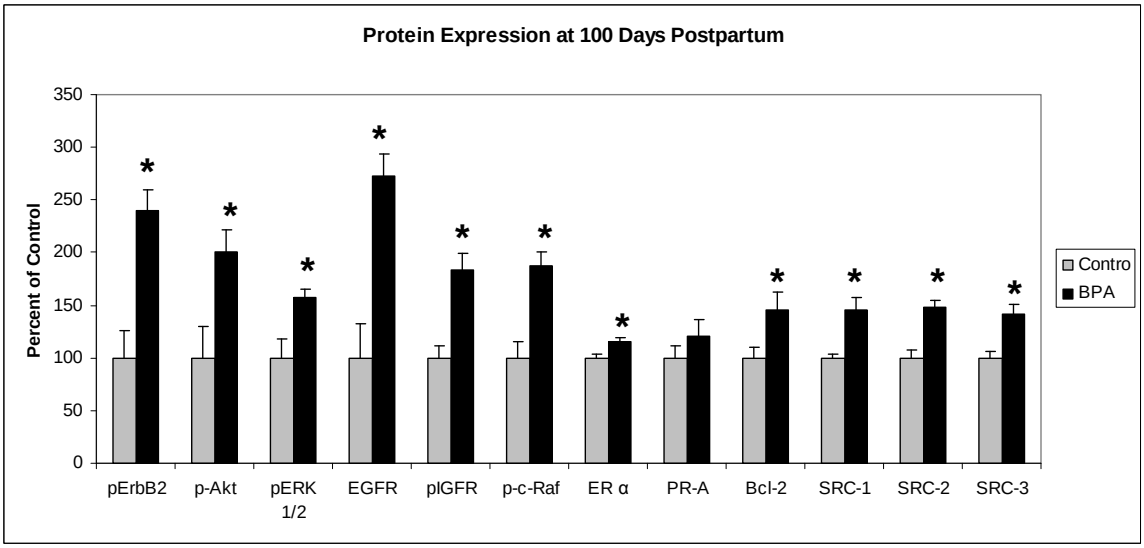

Protein Expression at 100 Days Postpartum
Percent of Control
350
300
250
200
150
100
50
0
pErbB2
p-Akt
pERK 1/2
EGFR
pIGFR
p-c-Raf
ER α
PR-A
Bcl-2
SRC-1
SRC-2
SRC-3
Contro
BPA

Figure 7:

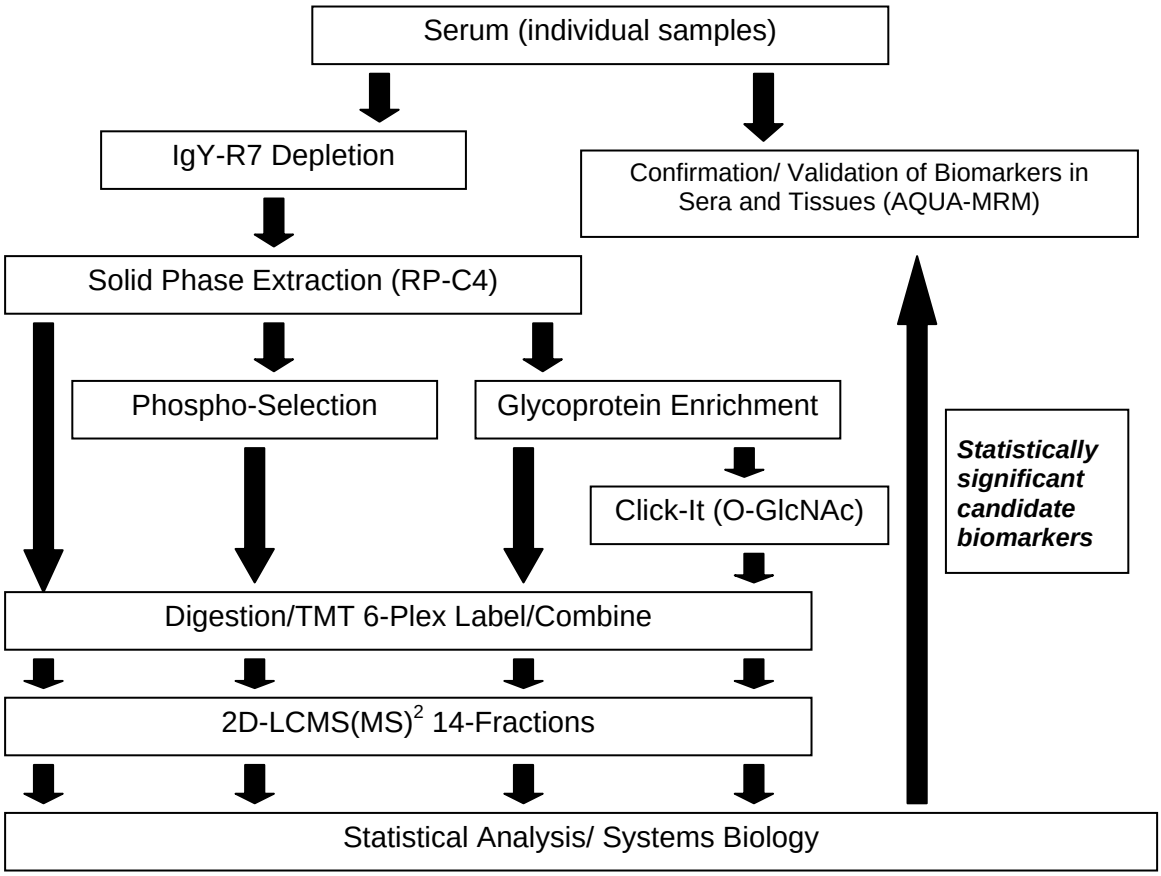
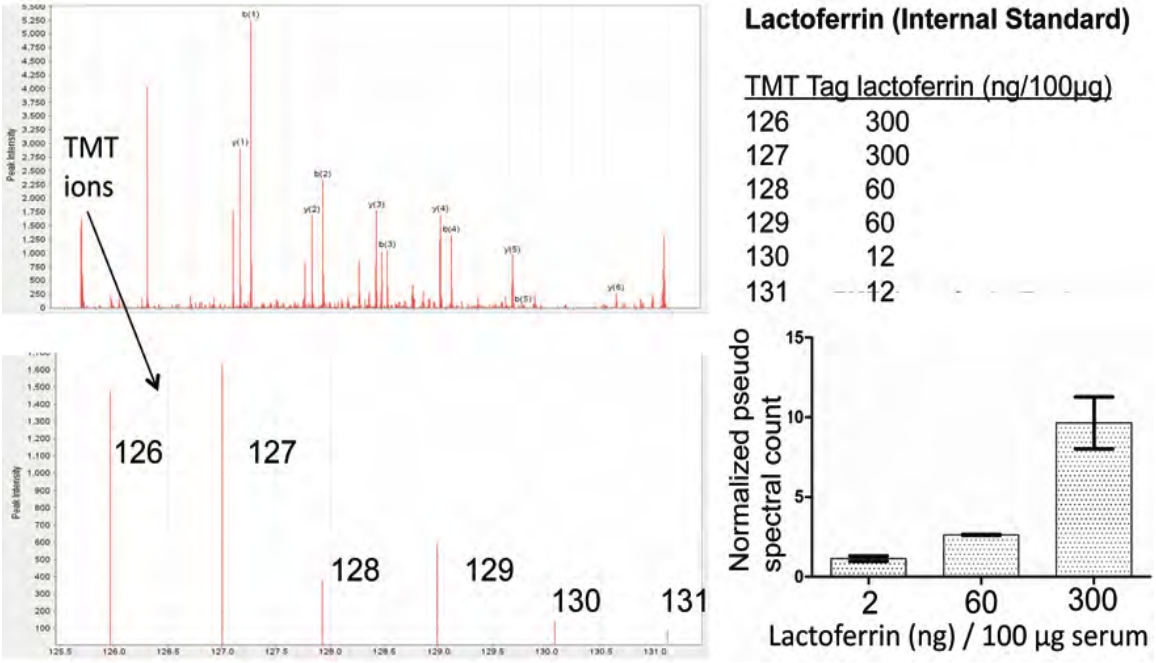

Serum (individual samples)
IgY-R7 Depletion
Confirmation/ Validation of Biomarkers in Sera and Tissues (AQUA-MRM)
Solid Phase Extraction (RP-C4)
Phospho-Selection
Glycoprotein Enrichment
Click-It (O-GlcNAc)
Statistically significant candidate biomarkers
Digestion/TMT 6-Plex Label/Combine
2D-LCMS(MS)2 14-Fractions
Statistical Analysis/ Systems Biology

Figure 8:

A.

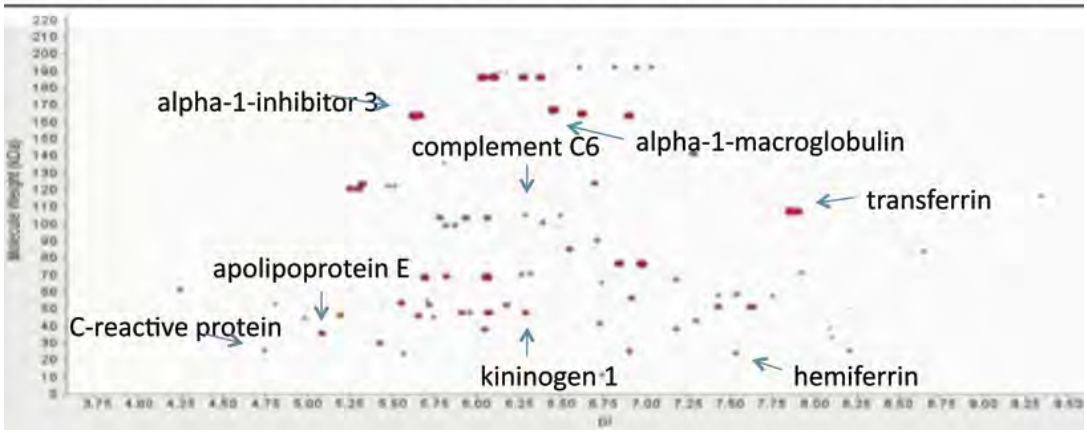


B.

Summary: (peptide & protein probability cut-off's set at 80% & 95% confidence)

number of peptides identified: 3032
number of unique peptides identified: 774
number of unique protein groups: 282
number of proteins with > 1 unique peptides 126
number of proteins with unique peptides carrying > 1 quantifiable mass tag: 87

A 2D Rendered Gel Plot of the 87 quantified proteins identified with high confidence:



APPENDIX C:

Jenkins S, Eltoum I, Desmond R, and Lamartiniere CA. Chronic Oral Exposure to Environmentally Relevant Concentrations of Bisphenol A Increases Mammary Carcinogenesis and Metastasis. **(In Submission)**

Chronic Oral Exposure to Environmentally Relevant Concentrations of Bisphenol A Increases Mammary Carcinogenesis and Metastasis

Sarah Jenkins[1], Jun Wang[1], Isam Eltoum[2,4], Rene Desmond[3,4], and Coral A. Lamartiniere[1,4]

[1]Department of Pharmacology and Toxicology, University of Alabama at Birmingham

[2]Department of Pathology, University of Alabama at Birmingham

[3]Department of Medicine, Division of Preventative Medicine, Biostatistics Unit, University of Alabama at Birmingham

[4]Comprehensive Cancer Center, University of Alabama at Birmingham

Corresponding Author: Coral A. Lamartiniere, Department of Pharmacology and Toxicology, University of Alabama at Birmingham, 1670 University Boulevard, Volker Hall L106, Birmingham AL 35294, E-mail: Coral@uab.edu Phone: (205) 934-7139; Fax: (205) 934-8240

Keywords: bisphenol A, BPA, oral, mammary gland, MMTV-erbB2, tumorigenesis, apoptosis

ABSTRACT

Bisphenol A (BPA) is a synthetically made compound used to produce a myriad of consumer goods. Recent studies found BPA to leach from these products in appreciable amounts, resulting in nearly ubiquitous daily exposure to humans. Whether BPA is harmful to humans, especially when administered in environmentally relevant concentrations via the oral route, is currently a topic of heated debate. Accordingly, this study investigated whether chronic, oral exposure to a range of BPA concentrations could alter spontaneously developing mammary cancer in mice that over-express the wild type erbB2/neu transgene (MMTV-erbB2). MMTV-erbB2 mice were provided drinking water containing 0, 2.5, 25, 250, or 2500 µg BPA/L from eight weeks of age until sacrifice. These concentrations resulted in daily exposure estimates spanning human relevance (2.5 and 25 µg BPA/L) to higher concentrations of toxicological and regulatory significance (250 and 2500 µg BPA/L). Interestingly, only the human relevant concentrations of BPA significantly decreased tumor latency and increased tumor multiplicity, burden, and the incidence of metastasis. Prior to tumor formation, all BPA doses significantly increased the cell proliferation index in the mammary gland. However, only the doses of toxicological significance also increased the apoptotic index in the mammary gland. We surmise that because the human relevant concentrations of BPA lacked a compensatory increase in the apoptotic index, the ratio of proliferating cells to apoptotic cells in the mammary gland was disrupted, which could contribute to carcinogenesis. This ratio best predicted the non-linear response of tumorigenesis evoked by BPA in the MMTV-erbB2 mouse model.

INTRODUCTION

The environmental contaminant, Bisphenol A (BPA), is used in the production of polycarbonate plastics and epoxy resins. These products are used to manufacture commonly used consumer goods such as food and beverage containers, the lacquer lining of canned foods and drinks, infant formula bottles, office water coolers, and some dental sealants. Several studies have found BPA to leach from these products in appreciable amounts, resulting in nearly ubiquitous daily exposure to humans (1, 2).

BPA has been classified as a xenoestrogen due to its ability to bind to the estrogen receptors (ER) and induce downstream transcriptional activity, albeit with an affinity several orders of magnitude less than estradiol (3-5). However, alternate targets have been identified, and the exact mechanism by which BPA functions is currently unknown (6-8). Most of the existing research has focused on the effects of short-term BPA exposure, administered during specific windows of early life development, and the resultant later life consequences. Collectively, these studies have shown that early life exposure to BPA in females rodents can alter the onset of puberty, disrupt estrous cyclicity and normal mammary gland development, and increase the development of preneoplastic lesions and neoplastic tumors in the mammary gland (Reviewed in (9)).

This study investigated the hypothesis that chronic, oral exposure to a range of BPA concentrations would result in a dose-dependent increase in the development and progression of mammary cancer in a mouse model that over-expresses the wild type erbB2/neu proto-oncogene. Interestingly, we found chronic BPA consumption to result in a non-linear dose response for mammary tumorigenesis and metastasis which was best

predicted by each treatment's alteration to the ratio of cell-proliferation-to-apoptosis in the mammary gland.

MATERIALS AND METHODS

Chemicals and Animals- BPA was purchased from Sigma Chemical Company (St. Louis, MO). Animal care and use were conducted according to established guidelines approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. A colony of MMTV-erbB2/neu transgenic mice (FVB/N-TgN(MMTV-neu202Mul)) was purchased from Jackson Laboratory (Bar Harbor, ME).

Tumorigenesis- Female MMTV-erbB2 mice were exposed to BPA *via* the drinking water from eight weeks of age until sacrifice (36 weeks of age). The following treatment groups were set up: 0 (control, *n*=94), 2.5 (BPA 2.5, *n*=38), 25 (BPA 25, *n*=76), 250 (BPA 250, *n*=37), and 2500 (BPA 2500, *n*=36) µg BPA/L drinking water. Figure 1 summarizes each of the doses used, the estimated daily intake, and the significance of each dose. All mice were palpated twice weekly and weighed monthly. Mice were sacrificed at 36 weeks of age or when tumors exceeded 10% body weight. All tumors, gross lesions, and lung lobes were fixed in formalin and blocked in paraffin for pathological evaluation (10). All tumors and lung metastases were evaluated by a board certified pathologist, Dr. Isam Eltoum.

Immunohistochemical Staining- Mice were exposed to 0 (*n*=14), 2.5 (*n*=11), 25 (*n*=17), 250(*n*=16), or 2500 (*n*=17) µg BPA/L drinking water beginning at eight weeks of age. At

16 weeks of age, the number four abdominal mammary glands were collected. Slides were boiled in citrate buffer, incubated in hydrogen peroxide, and blocked using normal horse serum. The slides were incubated in Ki-67 primary antibody (Dako, Denmark). Positively stained cells were visualized by 3,3'-dimonobenzidine (DAB) staining.

Apoptosis- The ApopTag Plus Peroxidase *in situ* Apoptosis Detection kit (Millipore, Billerica, MA) was used according to the manufacturer's protocol. Sections were pretreated with proteinase K, incubated in hydrogen peroxide, and equilibrated before terminal deoxynucleotideyl transferase enzyme was added. After enzyme incubation, anti-digoxigenin conjugate was added and the sections were incubated in DAB.

Statistical Analysis- The statistical method for evaluating tumor latency was performed as previously described (11). Tumor multiplicity was analyzed using the Cochran-Armitage trend test. The incidence of pulmonary metastasis was compared using Fisher's Exact test. Normally distributed data were analyzed using analysis of variance followed by Bonferroni's multiple comparison test (MCT). Data not normally distributed were analyzed by Kruskal-Wallis followed by Dunn's MCT. P-values ≤ 0.05 were considered statistically significant.

RESULTS

Only Human Relevant Concentrations of BPA Alter Tumorigenesis- Beginning at eight weeks of age, female MMTV-erbB2 mice were exposed to 0, 2.5, 25, 250, or 2500 µg BPA/L drinking water. These concentrations spanned from exposure estimates of human

relevancy (BPA 2.5 and BPA 25) to higher concentrations which define the upper limits of allowable exposure in the United States (BPA 250 and BPA 2500). The human relevant concentrations of BPA (BPA 2.5 and BPA 25) significantly increased tumor multiplicity, increasing the average number of tumors per mouse by 58% and 49%, respectively (Figure 2A). Human relevant concentrations of BPA also significantly decreased the time-to-first-tumor latency, reducing the time of tumor onset by over 29 days as compared to control (Figure 2B). The time-to-second- and time-to-third-tumor latencies followed similar decreases (data not shown). Tumor volume was also significantly increased following chronic exposure to BPA 25 and trended towards an increase with BPA 2.5 (Figure 2C). Further, chronic exposure to human relevant concentrations of BPA significantly increased the percentage of mice that developed pulmonary metastasis (Figure 2D). All of these effects were absent in the higher, toxicological-based doses of BPA. No significant differences between treatments were noted for the grade of developing tumors (data not shown).

Overt Markers of Toxicity- Only the toxicological-based doses of BPA 250 and BPA 2500 altered body weight (Table 1). Both of these losses represented reductions in body weight less than 10%, which failed to reach toxicological significance. BPA also caused a slight increase in uterine wet weight and uterine weight to body weight ratio, with BPA 250 achieving statistical significance (Table 1).

Cellular Proliferation and Apoptosis in the Mammary Gland- Previous reports have shown that early life exposure to BPA, during the perinatal period via subcutaneously

implanted pumps or during the prenatal/prepubertal period by intragastric gavage, can have long-lasting effects on cell turnover in the mammary gland (11-14). This information, coupled with the central role of cell turnover in carcinogenesis, led us to assess the cell proliferation and apoptotic indices in the mammary glands of mice chronically consuming BPA.

BPA resulted in a dose-dependent increase in the cell proliferation index at the human relevant doses of BPA before plateauing at the higher doses (Figure 3A). BPA exposure also caused an apparent dose-dependent increase in the apoptotic index in the mammary gland (Figure 3B). This resulted in the highest administered dose, BPA 2500, having a significantly higher apoptotic index as compared to control. When cell proliferation and apoptosis indices were taken together to estimate “cell turnover” in the mammary gland, the resultant cell-proliferation-to-apoptosis ratio showed a non-linear dose response curve that closely mimicked the tumorigenic response of MMTV-erbB2 mice following chronic exposure to BPA (Figure 3C). Both BPA 2.5 and BPA 25 resulted in proliferation-to-apoptosis ratios greater than control, with BPA 25 achieving statistical significance. The higher doses, BPA 250 and BPA 2500, failed to differ significantly from control.

DISCUSSION

Despite several countries altering regulations governing safe BPA intake, limitations in the United States have remained largely unchanged. This has been blamed on a lack of compelling evidence that BPA is capable of causing harm, citing study limitations in the existing literature such as inappropriate routes of administration,

utilizing too narrow of a concentration range, pursing end points not directly tied to pathology, and using statistically unsound sample sizes. Thus, the aim of this study was to evaluate the impact of chronic, oral BPA exposure on the development and progression of mammary carcinogenesis while addressing many of these aforementioned deficits.

Chronic administration of BPA resulted in a non-monotonic dose response for many of the tumorigenesis end points, with human-relevant concentrations of BPA decreasing the time to tumor onset and increasing tumor multiplicity, tumor volume, and the incidence of metastasis. While tumor grade failed to differ significantly between treatment groups, significant increases in tumor volume and the incidence of metastasis in mice chronically consuming human relevant concentrations of BPA support the recent study by Daikee et al. which suggested BPA may increase aggressive tumor formation (15). Furthermore, the regulatory-based concentrations of BPA failed to significantly alter any tumorigenesis end point. These data suggest that chronic BPA consumption during adulthood functions in a non-linear fashion for the development and progression of mammary carcinogenesis, wherein lower concentrations of BPA act more potently than higher doses, in the MMTV-erbB2 mouse model.

Non-monotonic dose response curves have been previously noted in the literature. Hugo et al. found that fat explants treated with BPA (0.1-10 nM) induced a “U-shaped” curve of adiponectin secretion, with low doses inhibiting and higher doses increasing adiponectin secretion (16). Using ICR mice, Miyawaki et al. administered 1 or 10 µg BPA/L drinking water from gestational day 10 until weaning. At the time of sacrifice, 1 µg, but not 10 µg, BPA/L significantly increased adipose tissue weight and the concentration of serum leptin as compared to control mice (17). As BPA has been

described as a xenoestrogen, it is interesting to note that a non-monotonic dose response has also been reported for estradiol. Vandenberg et al. found that mice exposed to estradiol exhibited a non-monotonic dose response for morphology parameters of mammary gland development (18). However, while it may be convenient to attribute the non-monotonic behavior of BPA on its ability to act as a weak estrogen, our experiments showed only slight changes in uterine wet weights, a parameter classically used to estimate a compound's estrogenic potential. Thus, it becomes interesting to note that other classes of compounds, most notably the angiogenesis inhibitors, have also reported non-monotonic therapeutic dose responses (19, 20). Given that our data suggest a role for BPA in tumor angiogenesis (significant increases in tumor volume and the percent of animals developing pulmonary metastasis), future research efforts focusing this connection are warranted.

Currently, very little to no empirical evidence exists which explains the physiological basis of the non-monotonic dose response. To this end, we focused our attention on investigating potential mechanisms behind this response in our model. Overt markers of toxicity and estrogenic activity indicated only dose-dependent alterations that resulted in minor effects at the highest concentrations studied. This confirmed much of the literature which report BPA as a weak estrogen. At the cellular level, chronic BPA administration caused dose-dependent increases in the cell proliferation and apoptosis indices in the mammary gland. Several studies investigating the effects of early life exposure to BPA in the mammary gland have shown similar results (11-14). When the cell proliferation indices and the apoptotic indices were combined to produce an estimation of "cell turnover" in the mammary gland, a non-monotonic dose response

curve resulted. It was this end point that best predicted the ability of each concentration of BPA to alter tumorigenesis in this model.

Our data suggest that the observed non-montonic dose response observed for tumorigenesis is due, at least in part, to the differing ability of each concentration of BPA to induce apoptosis. All of the BPA concentrations studied were capable of increasing cell proliferation. However, only the regulatory-based concentrations of BPA were capable of countering the increased cell proliferation with a simultaneous increase in apoptosis in the mammary gland. As a result, neither of the regulatory-based doses produced a ratio of "cell turnover" that differed significantly from control treated mice. Conversely, without an increase in apoptosis, human-relevant concentrations of BPA resulted in a mammary gland where high rates of proliferation were coupled to low rates of apoptosis, skewing "normal" cell turnover and potentially contributing to carcinogenesis. Future research should be directed towards investigating proteins that play a role in regulating apoptosis in the mammary gland and the measurement of BPA blood and urine concentrations in similarly treated animals.

In summary, these data provide the first evidence that clearly demonstrates the potential of chronic, oral administration of human-relevant concentrations of BPA to impact mammary tumorigenesis in a non-monotonic fashion. While it is not clear whether outcomes modeled in animals recapitulate the human situation exactly, these data strongly support the existing literature which suggest that even "low" exposures to the environmental contaminant BPA may be capable of harm.

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Competing interests: The authors do not have any conflict of interest to report.

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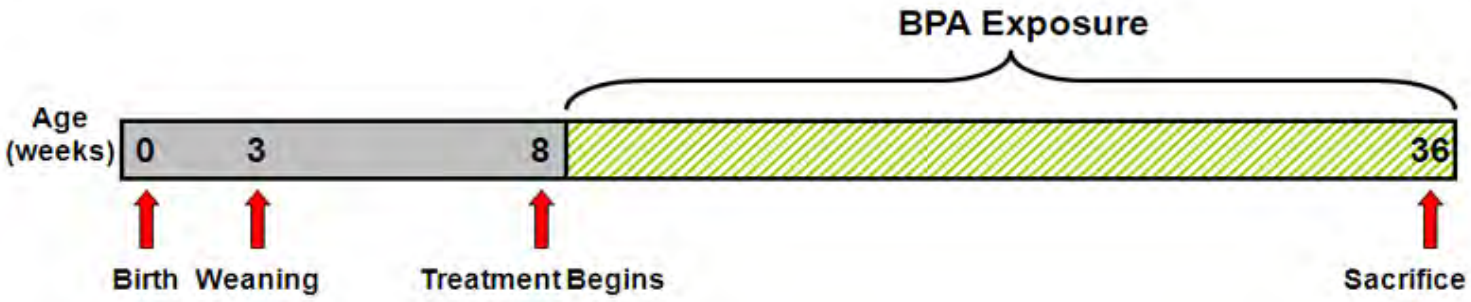


Groups	Treatment	Estimated Intake	Significance
Control (*n*=94)	0 µg BPA/L	0 µg BPA/kg BW	Baseline
BPA 2.5 (*n*=38)	2.5 µg BPA/L	0.5 µg BPA/kg BW	Human Relevant Exposure
BPA 25 (*n*=76)	25 µg BPA/L	5 µg BPA/kg BW	High Human Relevant Exposure
BPA 250 (*n*=37)	250 µg BPA/L	50 µg BPA/kg BW	EPA's Reference Dose
BPA 2500 (*n*=36)	2500 µg BPA/L	500 µg BPA/kg BW	1% LOAEL

Figure 1: Lifetime BPA Dosing Schematic. Beginning at eight weeks of age, female MMTV-erbB2 mice were provided 0, 2.5, 25, 250, or 2500 µg BPA/L drinking water. Estimated daily intakes were calculated on the basis of a 20 gram mouse drinking four milliliters of water daily.

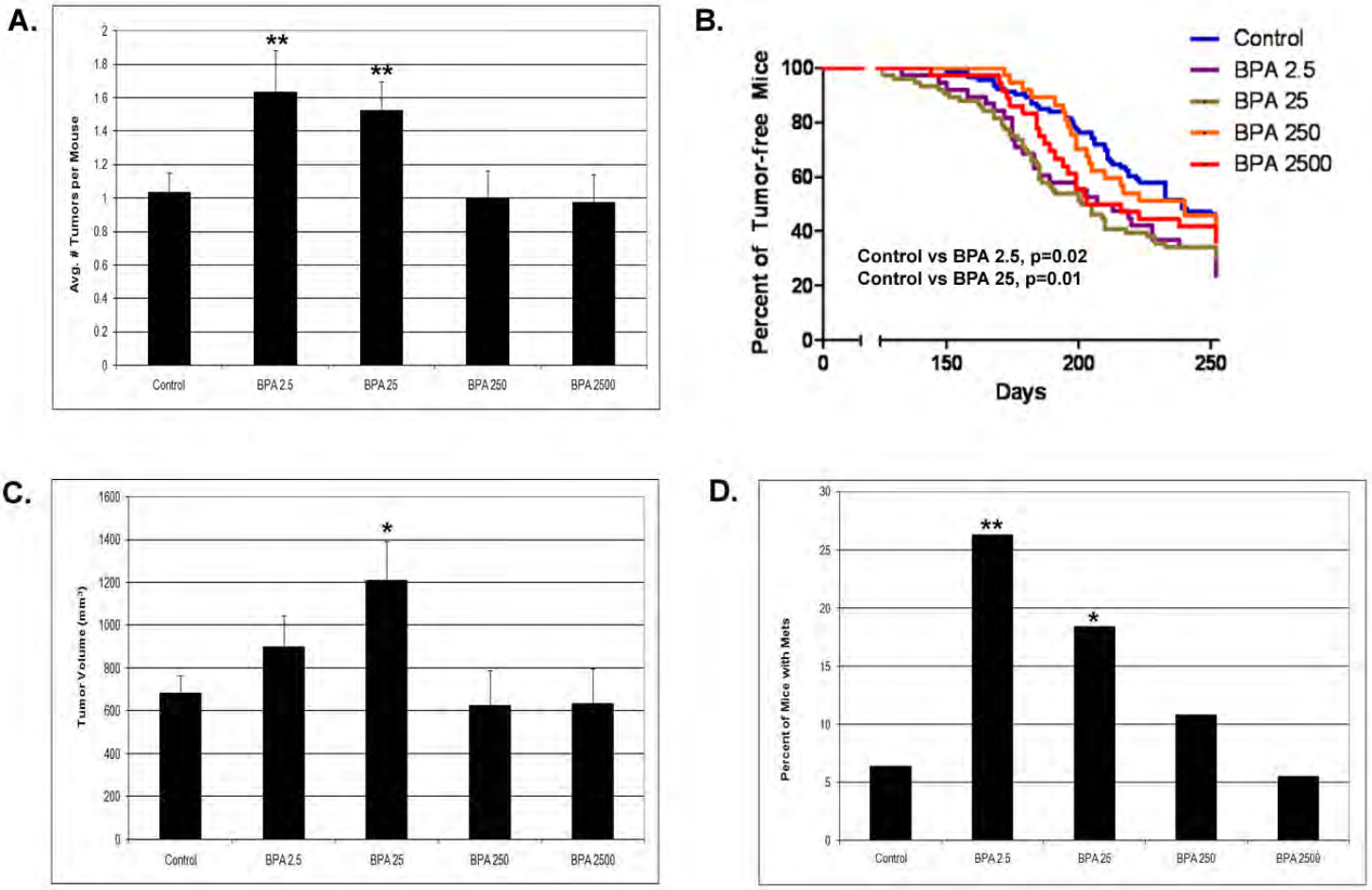
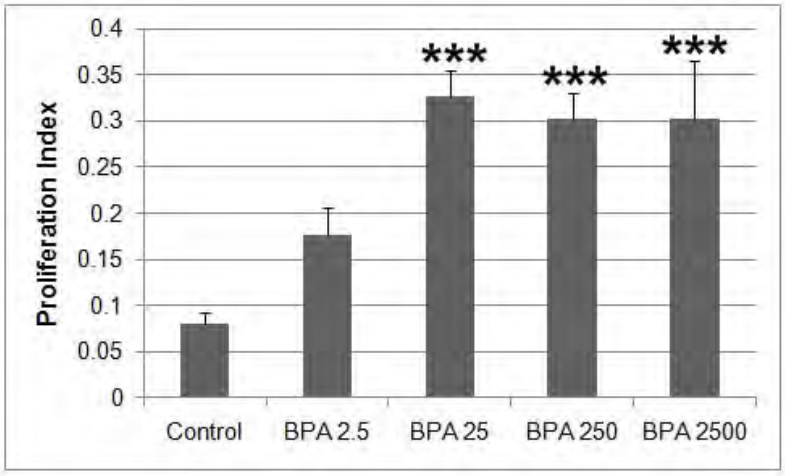
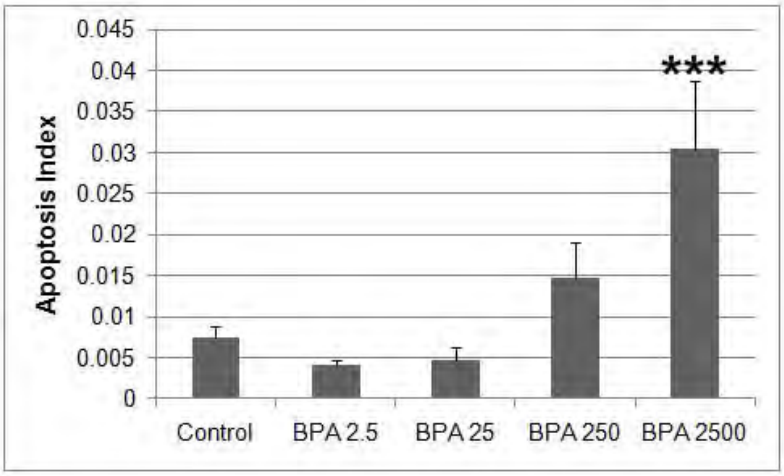


Figure 2: (A) Tumor multiplicity, (B) time to first tumor latency, (C) tumor volume, and (D) incidence of pulmonary metastasis in MMTV-erbB2 mice following chronic oral exposure to BPA. Values in tumor multiplicity and tumor volume represent average values ± standard error. As compared to control, * represents a p-value <0.05, and ** represents a p-value <0.01.

A.


Proliferation Index
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
***
***
***
Control
BPA 2.5
BPA 25
BPA 250
BPA 2500

B.


Apoptosis Index
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
***
Control
BPA 2.5
BPA 25
BPA 250
BPA 2500

C.

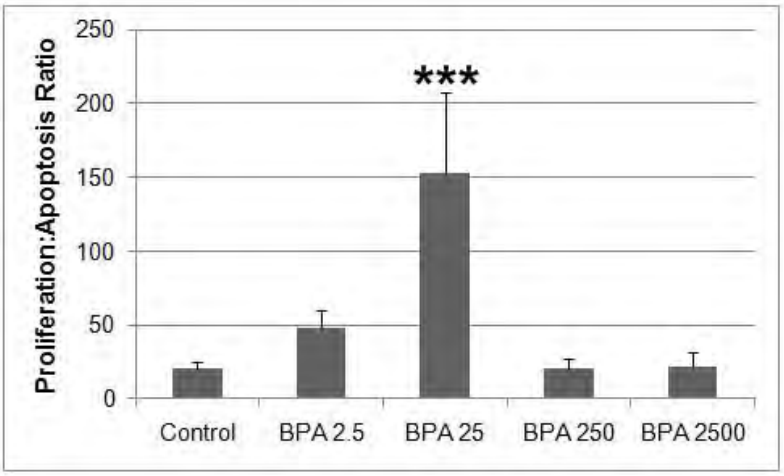

Proliferation:Apoptosis Ratio
250
200
150
100
50
0
***
Control
BPA 2.5
BPA 25
BPA 250
BPA 2500

Figure 3: The (A) proliferation index, (B) apoptotic index, and (C) proliferation-to-apoptosis ratio in the mammary glands of mice treated ± chronic, oral exposure to BPA. Values represent average values ± standard error. As compared to control, *** represents a p-value <0.001.

Treatment	Body Weight (g)	Uterine Weight (mg)	Uterine:BW Ratio
Control	26.0 ± 0.3	90.2 ± 4.3	3.5 ± 0.2
BPA 2.5	25.3 ± 0.6	100.7 ± 7.2	4.0 ± 0.3
BPA 25	25.9 ± 0.4	107.3 ± 6.0	4.2 ± 0.2
BPA 250	24.1 ± 0.2*	123.3 ± 13.6*	5.1 ± 0.6***
BPA 2500	24.4 ± 0.3	113.0 ± 6.5	4.6 ± 0.3

Table 1. Body weight, uterine wet weight, and uterine-to-body-weight ratio in MMTV-erbB2 mice following chronic oral exposure to BPA. Values represent averages ± standard error. As compared to control, * indicates a p-value <0.05, and *** indicates a p-value <0.001.